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BLOOD VOLUME STUDIES

I. Experimental Control of a Dye Blood Volume Method

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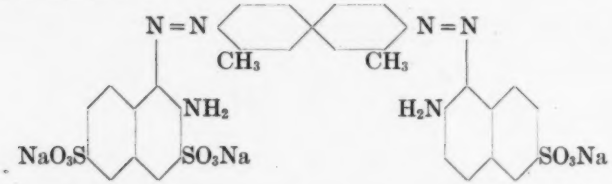
Studies of blood volume developed in this laboratory as the result of work which was being done in the field of pigment metabolism. Both bile pigment and blood pigment were being studied with particular regard to reciprocal pigment production and destruction in the bile and blood respectively (1). It is obvious that no comprehensive study of blood pigment can be made without reasonably accurate determinations of blood volume. It is remarkable how much effort has been expended in a study of cell counts and hematocrit readings under various experimental conditions with no knowledge concerning the blood volume figures. If a critic chooses to assume any fluctuations in blood volume, all the carefully drawn hypotheses in such papers must become valueless. That such fluctuations in blood volume do occur following a variety of experimental conditions will be shown in experiments to be published in this series of papers.

The final blood volume method as outlined in this paper is the end result of a series of modifications made in the original dye method published by Keith, Rowntree and Geraghty (2). Vital red, the dye used in their experiments, was not obtainable in sufficient quantity and it was necessary to substitute the dye "brilliant vital red." Dr. Herbert M. Evans, Professor of Anatomy, with his expert knowledge of dyes, when consulted concerning the use of other dyes in the estimation of plasma volume, suggested "brilliant vital red." This opportunity is taken to express appreciation of Doctor Evans' interest in

this work and the valuable assistance and materials which he has given so generously.

"Brilliant vital red" is tolidine combined with one molecule of β naphtylamine, 3,6 disulfonic acid and one molecule of β naphtylamine 6 monosulfonic acid.



It is readily soluble in water and does not dialyze against water through celloidin sacs. It is harmless when injected intravenously in the amounts used for the estimation of plasma volume. In none of the many experiments with the blood volume method which have been performed by us have any symptoms been noted that could be attributed to toxicity on the part of the dye. To further test this point 20 cc. of a 1 per cent solution of brilliant vital red were injected into the jugular vein of a mongrel bull pup weighing 6.6 kilos. Although this is approximately fifteen times the amount injected in a blood volume determination no evil effects were observed except a rise of 0.7°C. in temperature for a period of two hours. There is no evidence that the dye has any hemolytic action on normal dog's blood.

METHOD

Normal dogs were used in all these experiments. A hypodermic needle is inserted into the vein and 10 cc. of blood drawn (with as little compression of the vein as possible) into a dry well-vaselined syringe. The blood is immediately placed in a 15 cc. graduated hematocrit tube containing 2 cc. of a 1.6 per cent solution of sodium oxalate. The blood and oxalate are mixed by inversion and the tube stoppered. A standard amount of the dye is drawn up into a syringe along with 5 to 10 cc. of 0.9 per cent saline. This dye solution is now injected into the jugular vein, the dye being washed completely out of the syringe into the blood stream by means of a few cubic centimeters of saline. The dye solution is 1 per cent strength and is given in the amount of 1 cc. per 5 kilos body weight. This is the amount arbitrarily taken by us but more or less may be used to suit individual taste in color readings.

Exactly four minutes after the injection of this dye a clean needle is again inserted into the jugular vein (preferably of the opposite side) and another 10 cc. sample of blood drawn and placed in another hematocrit tube also containing 2 cc. of 1.6 per cent sodium oxalate. The hematocrit tubes are now centrifugalized at 2500 revolutions a minute for 30 minutes. The total contents of the tube and the number of cubic centimeters of blood cells are now noted. Two cubic centimeters of the dye-colored plasma are pipetted off and diluted in a small tube with 4 cc. of 0.9 per cent sodium chloride. This unknown is then read in a colorimeter against a standard prepared as follows:

1. Seventy-five-hundredths cubic centimeters of 1 per cent brilliant red vital is pipetted into a 200 cc. volumetric flask which is then made up to the mark with distilled water.

2. Five cubic centimeters of this aqueous dye solution are then diluted with 5 cc. of clear dye-free plasma (obtained from the first sample of blood drawn from the animal) and 5 cc. of 0.9 per cent sodium chloride, making in all 15 cc. of standard dye solution.

Against this standard the above unknown is read and expressed in per cent. This value will henceforth be referred to as R.

Let us assign the following values to be used in the formula below:

D equals the number of cubic centimeters of 1 per cent brilliant vital red injected into the animal.

C, the correction for the dilution by oxalate present equals the total number of cubic centimeters of oxalated plasma present in the second sample of blood drawn, minus 2 divided by the total number of cubic centimeters of oxalated plasma present in the same tube. This value expresses the ratio between the actual concentration of dye in the plasma when diluted with oxalate solution to the value when not so diluted.

R equals the observed colorimetric reading (in per cent of the standard).

Plasma per cent means the percentage of the whole blood which the plasma constitutes, and is obtained by dividing the total number of cubic centimeters of oxalated plasma present in the hematocrit tube minus 2, by the total contents of the tube in cubic centimeters minus 2. Then

$$\text{The plasma volume (in cc.)} = \frac{26666.67\ D\ C}{R}$$

$$\text{The blood volume} = \frac{\text{Plasma Volume} \times 100}{\text{Plasma Per Cent}}$$

The above formula for plasma volume may be derived as follows: The standard for color comparison contains 0.75 cc. of 1 per cent dye in 200 cc. of fluid, or 1 cc. in 266.66+ cc. D cc. of 1 per cent dye (the amount injected) will impart the same color intensity to 266.6667 D cc. of fluid. If, however, the color intensity is $\frac{R}{100\ C}$, (i.e., the colorimetric reading in per cent corrected for the dilution of the plasma by the oxalate solution), the number of cubic centimeters of fluids equals $\frac{(266.6667D)\ (100C)}{R}$ or $\frac{26666.67\ D\ C.}{R}$

METHOD CONTROLS

In the method as outlined above the blood drawn from the animal is mixed with a known amount of an isotonic solution of sodium oxalate (see below) instead of with solid sodium oxalate as was done by Keith, Rowntree and Geraghty (2). The experiments cited in table 1 illustrate the fact that shrinking of blood cells results when even small amounts of solid sodium oxalate are added. Thus the minimal amount of oxalate which can be used (10 mgm. to 10 cc. of blood) causes on an average a shrinkage of about 3 per cent in the cell volume. In actual practice a considerable excess of oxalate may be added unless carefully weighed out. In such cases the error is still larger and may in fact be several times this size.

To obtain the true hematocrit value the anticoagulant used must be isotonic with blood. It is true that samples of blood drawn from different individuals differ from each other in tonicity, but the variation is very slight except in certain pathological conditions such as uremia. The tonicity of normal blood is generally stated to be equal to that of a 0.95 per cent solution of sodium chloride. As far as we have been able to learn, no accurate physical-chemical investigations have been made to determine the concentration of sodium oxalate isotonic with physiological sodium chloride. Assuming that the dissociations of sodium and potassium oxalates are not very far different we find by calculation from the tables of Noyes and Johnston (3) that a 1.6 or 1.7 per cent solution of sodium oxalate would be approximately isotonic with a 0.95 per cent solution of sodium chloride. Valuable advice covering this physical-chemical work was kindly rendered us by Dr. Carl L. A. Schmidt of the Department of Biochemistry.

A number of experiments have been carried out to determine by *biological tests* that concentration of sodium oxalate which, placed in contact with normal red blood cells, produces neither shrinking nor swelling of these cells. Table 2 below shows the results of two experiments on defibrinated normal dog's blood. In both cases the 1.6 per cent solution of sodium oxalate is approximately isotonic. The effect of oxalate solutions on the blood cells of peptonized blood is given in the third column of the same table. 1.4 per cent is obviously hypotonic, while 1.7 per cent is hypertonic. By interpolation the point of isotonicity is found to be about 1.5 per cent. We may, then, from the physical-chemical data supported by biological experiments, conclude that 1.6 per cent sodium oxalate is approximately isotonic with normal dog's blood.

Experiment 222. Tonicity of solutions of sodium oxalate

Two hundred and thirty cubic centimeters of blood were rapidly aspirated from the jugular vein of a normal 40-pound dog (17–160). This blood was immediately defibrinated by whipping.

Two cubic centimeters of sodium oxalate solutions of varying concentrations were pipetted into each of a series of seven tubes. The eighth was left empty. A second and identical series was set up as duplicates. Into each of these tubes were pipetted 9 to 10 cc. of the well-mixed defibrinated blood. The contents of the tubes were mixed, the tubes corked and centrifugalized for one-half hour at 3000 revolutions per minute. The quantity of packed cells and of supernatant fluid was read off on each of the tubes. After making proper allowance for the quantity of fluid added along with the oxalate, the per cent of cells was calculated. The averages for the duplicates are given in table 2.

Identically the same procedure was carried out on a similar quantity of blood obtained from another normal 42-pound dog (621). The results are given also in table 2.

Experiment 224. Tonicity of solutions of sodium oxalate

Three grams of "Witte's Peptone" dissolved in 20 cc. of Locke's solution were injected rapidly into the jugular vein of a normal young 10-pound female terrier under complete ether anesthesia. After 30 minutes a cannula was placed in the carotid artery and samples of blood were drawn into each of a series of four hematocrit tubes. Of these tubes three contained each 2 cc. of solutions of sodium oxalate of varying concentration. The fourth tube contained no oxalate and was prevented from clotting by the familiar "peptone reaction." This experiment supplements the data furnished in the two defibrination experiments. All of the tubes were then centrifugalized simultaneously for 30 minutes at 3000 revolutions per minute. The quantity of packed cells and of supernatant fluid was read off on each of the tubes. After making proper allowance for the quantity of fluid added along with the oxalate, the per cent of cells was calculated. The results are given in table 2.

Dye removed by blood coagulation. When the error due to the use of dry powdered oxalate was first appreciated we turned to the use of blood serum obtained by coagulation in a vaselined tube. The blood was obtained as usual four minutes after dye injection, allowed to clot

TABLE 1

Red blood cell hematocrit modified by solid sodium oxalate

DOG	QUANTITY OF DEFIBRINATED BLOOD	HEMATOCRIT (IN PER CENT RED BLOOD CELLS) ON ADDING SOLID SODIUM OXALATE						
		0 mgm.	10 mgm.	20 mgm.	30 mgm.	50 mgm.	70 mgm.	100 mgm.
	cc.							
19–128	10	57.9	55.0	52.0	49.5	43.2	45.6	42.7
17–160	10	57.5	54.5	53.0	50.0	49.5	46.2	44.0
19–106	10	49.8	46.7	45.7	43.8	44.3	43.4	40.0

A fair approximation of the usual amount of dry powdered oxalate used in routine work may be placed at 40 to 60 mgm. per 10 cc. blood.

TABLE 2

Tonicity of solutions of sodium oxalate

PER CENT SODIUM OXALATE USED	HEMATOCRIT BLOOD CELL PER CENT		
	Experiment 222, dog 17-160	Experiment 222, dog 621	Experiment 224, dog 19-107, young adult
1.0	48.0	63.0	63.7
1.1	47.7	62.0	
1.2	47.4	61.3	
1.3	46.6	61.2	
1.4	46.1	59.9	61.1
1.5	46.3	60.4	
1.6	45.2	59.1	
Control with no oxalate	45.1	58.9	59.6
1.7			56.6
	Defibrination of blood	Defibrination of blood	Peptone shock blood incoagulable

and the serum collected after centrifugalization. This serum was then compared colorimetrically with a known dye standard. Such steps would simplify the procedure as it would then be unnecessary to allow for dilution by the oxalate solution.

This method has been employed as a routine procedure in a large number of experiments which have been performed in this laboratory. Experiments cited below show that certain dangers are involved. Thus while experimenting with a recently purchased sample of brilliant vital red obtained from an English manufacturer it was found that in the process of clotting a certain amount of dye was removed from solution. This is shown in experiment 219. On an average in the three cases here cited about 7 per cent of the dye was removed from the plasma during the process of clotting. For this reason the blood volume values are too high when the serum is used for the dye reading.

The stock dye which has been used in the previous work was tested in a similar series of experiments by Mrs. F. S. Robscheit in this laboratory. In the case of this dye apparently none of the dye was so removed. In several tests made with this same dye we have confirmed this observation. The routine blood volume work already done in this laboratory with this serum method was all done with the old stock dye and therefore cannot be criticised from this standpoint. It is noteworthy that the dye obtained from the English manufacturer was of a different shade of red from the samples which we had previously used. Furthermore it was decidedly weaker in strength so that it was necessary to use a 2 per cent solution for injection instead of the customary 1 per cent solution.

Whether all samples of brilliant vital red when present in larger amounts (as in some of the experiments cited in the following paper) would be removed in the process of clotting is not as yet known. In view of this uncertainty and in view also of the differences in dyes obtained from different manufacturers we feel that to avoid possible error all samples should be collected in isotonic oxalate solution in the manner already described.

Experiment 219. The effect of blood clotting on the concentration of dye in the plasma

Three healthy young adult dogs were used in the following experiment:
Dog 19–38. Female Coach dog. Weight 24.68 pounds.
Dog 19–39. Female mongrel terrier. Weight 24.25 pounds.
Dog 18–92. Male mongrel terrier. Weight 18.75 pounds.

One cubic centimeter of brilliant vital red (English) for each five kilos body weight was injected intravenously. The blood volume determination was carried out in the usual way with the exception that in addition to the ordinary hematocrit sample taken four minutes after the injection of the dye, a 10 cc. sample was also drawn at this time into a dry vaseline-lined heavy-walled test tube and allowed to stand 30 minutes. At the end of this time the clot was

freed from the wall of the tube with a piece of wire. After centrifugalization (30 minutes) the serum was drawn off and diluted with two volumes of 0.9 per cent sodium chloride solution. The dye-colored oxalated plasma was obtained from the hematocrit tube in the same way and was similarly diluted. Both of these diluted dye-containing samples were then read colorimetrically against a standard prepared as follows: 0.5 cc. of the 2 per cent brilliant vital red was accurately brought up to 100 cc. in a volumetric flask; 5 cc. of this mixture were mixed with 5 cc. of dye-free plasma and 5 cc. of 0.9 per cent sodium chloride.

The observed readings and estimations for plasma volume and blood volume for each of the three dogs are given in table 3 below. It will be observed that the samples were read against a standard which is stronger than usual. This must be taken into account in making the plasma volume and blood volume calculations.

TABLE 3

Experiment 219. The effect of blood clotting on the concentration of dye in the plasma

SPECIMEN READ AGAINST STANDARD	OBSERVED READING AGAINST STANDARD			ESTIMATED PLASMA VOLUME			HEMATOCRIT (CELLS)			ESTIMATED BLOOD VOLUME		
	Dog 19-38	Dog 19-39	Dog 18-92	Dog 19-38	Dog 19-39	Dog 18-92	Dog 19-38	Dog 19-39	Dog 18-92	Dog 19-38	Dog 19-39	Dog 18-92
	per cent	*per cent*	*per cent*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*cc.*	*cc.*	*cc.*
Serum*.....	92	83	64	487	530	533	(52.1)	(60.5)	(48.2)	1017	1342	1029
Oxalated plasma (corrected for the dilution by the oxalate solution)	99	91	70	453	485	487	52.1	60.5	48.2	946	1228	940

*This indicates that blood coagulation removed a definite per cent of this dye from the plasma. For this reason the blood volume values are too high when the serum is used for the dye readings.

Centrifugalization factors. In order to determine the minimum length of time that is required to cause by centrifugalization at 2500 revolutions a minute a completion of the process of sedimentation, a number of 15 cc. hematocrit tubes filled with oxalated dog's blood were centrifugalized varying lengths of time. The distance of the bottom of the tube from the center of the circle of rotation equals 27 cm. Twenty minutes were found to be insufficient; however, the packing at the end of thirty minutes was in most cases as complete as at any subsequent time. According to Köppe (4) complete packing of the cells is accompanied by a translucency of the layer of packed cells. Such translucency according to him occurs only if the tubes are centrifugalized at a speed

of not less than 5000 revolutions a minute. The experiments no. 253 and 268 presented below show that if any of the dye-colored plasma remains in the interstices it is so small in amount as to be negligible. Moreover, we have performed experiments which show that packed cells from dye-containing blood when washed with very small amounts of isotonic saline impart to this saline only an exceedingly small trace of dye. For all practical purposes therefore the packing may be considered complete at the end of thirty minutes.

Experiment 268. The principle of the blood volume method tested in vitro

A total of 1500 cc. of blood was drawn from the jugular veins of four normal dogs into 75 cc. of 1.6 per cent sodium oxalate. This blood was thoroughly mixed. Nine hundred cubic centimeters were poured into a dry 1000 cc. volumetric flask. Two cubic centimeters of 1 per cent brilliant vital red were added and the flask made up to mark with oxalated blood. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time 14 cc. were quickly pipetted into a dry 15 cc. graduated hematocrit tube. This tube of blood was centrifugalized for 30 minutes at 3000 revolutions per minute. The quantity of packed cells and of supernatant fluid was then read off and the per cent of blood cells calculated to be 53.5 per cent.

Two cubic centimeters of the dye-tinged supernatant fluid were mixed with 4 cc. of 0.9 per cent NaCl and read against a standard prepared as follows: 0.75 cc. of 1 per cent brilliant vital red was accurately brought up to 200 cc. with distilled water in a volumetric flask. Five cubic centimeters of this were mixed with 5 cc. of the clear dye-free oxalated plasma and 5 cc. of the 0.9 per cent saline. Against this standard the above diluted sample of dye-colored plasma reads 117.3 per cent.

With this concentration of dye the number of cubic centimeters of oxalated plasma in the total 1000 cc. of blood would, from the previously given formula, be $\frac{26666.67 \times 2}{117.3} = 455$. The actual amount of oxalated plasma present (as indicated by the hematocrit) was 465 cc.

Experiment 253. Blood volume in vitro

Three hundred cubic centimeters of dog's blood were drawn into a bottle containing 35 cc. of 1.6 per cent sodium oxalate. This blood was thoroughly mixed. Two hundred cubic centimeters were poured into a dry 250 cc. volumetric flask, 0.455 cc. of 1 per cent brilliant vital red was added and the flask made up to mark with oxalated blood. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time two graduated 15 cc. centrifuge tubes were filled, allowed to stand for several minutes and centrifugalized at 2500 revolutions a minute for 30 minutes. The quantity of packed cells and of supernatant fluid was then read off and the per cent of blood cells calculated to be 53.9 per cent.

Two cubic centimeters of the dye-tinged supernatant fluid were mixed with 4 cc. of 0.9 per cent NaCl and read against a standard prepared as follows: 0.75 cc. of 1 per cent brilliant vital red was accurately brought up to 200 cc. with distilled water in a volumetric flask. Five cubic centimeters of this were mixed with 5 cc. of the clear dye-free oxalated plasma (obtained by centrifugalization) and 5 cc. of the 0.9 per cent NaCl. Against this standard the above diluted sample of dye-colored plasma read 100 per cent.

With this concentration of dye the number of cubic centimeters of oxalated plasma in the total 250 cc. of oxalated blood would, from the previously given formula, be $\frac{26666.67 \times 0.455}{100} = 121$. The actual amount present, as indicated by the hematocrit, was 115 cc.

Blood volume method tested in vitro. The dye method for the determination of blood volume has been tested *in vitro* on large quantities of oxalated

TABLE 4

Rate of elimination of brilliant vital red from circulation

BLOOD FROM DOG NUMBER	ACTUALLY OBSERVED COLORIMETRIC READING OF THE TWO MINUTE SAMPLE CORRECTED FOR OXALATE	RELATIVE CONCENTRATION OF DYE IN PLASMA AFTER INJECTION OF DYE (TWO MINUTE SAMPLE TAKEN AS 100)				
		Two minutes after injection	Four minutes after injection	Twenty minutes after injection	Three hours after injection	Twenty-four hours after injection
18–39	103.6	100	99.2	93.9		10 to 15
18–28	122.2	100	99.3	89.0	62.4	10 to 15
19–123	136.0	100	102.6	93.0	64.0	10 to 15
19–84	138.3	100	98.3	91.1	59.8	10 to 15
Average..	125.0	100	99.9	91.8	62.1	10 to 15

dog's blood in the experiments given above (268 and 253). The theoretical and the estimated quantity of oxalated plasma in the flask in one case differ by about 5 per cent; in the other by less than 2 per cent. The dye therefore is not absorbed to any appreciable extent by the blood cells nor is it to be found to any appreciable extent in the interstices between the blood cells when the latter are packed by the degree of centrifugalization employed. Furthermore, experiments have shown that no appreciable amount of dye could be extracted from this layer of packed cells by washing with small amounts of physiological saline.

Elimination of the dye from circulation. Any blood volume method based on the dilution of a substance introduced into the blood stream depends for its accuracy on a slow rate of elimination from circulation. The rate of elimination of brilliant vital red is shown in table 4. In this table are summarized experiments on four normal active young adult

dogs. An amount of dye equal to that customarily used in blood volume estimation was injected into each dog in the customary way. Ten cubic centimeter samples were collected at intervals after the injection of the dye. As can be seen from table 4, the concentration of the dye in the plasma in most cases is the same at the end of two minutes as at

TABLE 5

EXPERIMENT NUMBER	SEX	DESCRIPTION	WEIGHT	BLOOD VOLUME	PLASMA VOLUME	TOTAL BLOOD CELL VOLUME	HEMATOCRIT CELLS	BLOOD PER 100 GRAM BODY WEIGHT
			kgm.				*per cent*	*cc.*
305			7.27	640	310	330	51.6	8.80
233	f	Fox	8.52	892	365	527	59.1	10.47
237	m	Fox	9.09	1041	478	563	54.1	11.45
230	f	Coach	10.97	1197	601	596	49.8	10.91
235	f	Mongrel Spitz	11.14	1255	557	698	55.6	11.27
251	m	Mongrel	13.18	1357	643	714	52.6	10.30
257	m	Mongrel	13.18	1293	742	551	42.6	9.81
258	m	Spitz	13.18	1103	502	601	54.5	8.37
234		Mongrel	13.64	1439	525	914	63.5	10.55
261	m	Mongrel	13.64	1225	767	458	37.8	8.98
256	m	Mongrel	13.86	1314	644	670	51.0	9.48
211	m	Setter	15.91	1835	936	899	49.0	11.53
262	m	Bull	16.36	1487	769	718	48.3	9.09
240	f	Bull-terrier	17.73	1867	788	1079	57.8	10.53
241	m	Bull	17.73	1730	865	865	50.0	9.76
249	m	Setter	17.73	1867	958	909	48.7	10.53
247	m	Spaniel	18.41	2080	1029	1051	50.5	11.30
231	m	Shepherd	18.73	1614	1069	545	34.4	8.62
254	m	Shepherd	20.00	1955	919	1036	53.0	9.78
299			20.00	2119	945	1174	55.4	10.60
236	f	Terrier	20.57	2264	840	1424	62.9	11.01
238	m	Mongrel	22.05	2166	1025	1141	53.6	9.82
Average of dogs weighing from 7 to 15 kgm.			11.61	1160	558	602	52.0	10.04
Average of dogs weighing from 15 to 23 kgm.			18.65	1908	922	986	51.2	10.23
Average of all dogs			15.13	1538	740	794	51.6	10.13

the end of four minutes. At the end of twenty minutes, however, about 6 to 11 per cent is lost while in twenty-four hours only about 10 per cent remains. Blood volume estimations based on samples collected at the four-minute interval cannot therefore be invalidated because of the elimination of dye from circulation. It seems probable that the mixing of dye with the plasma is complete at the end of four minutes.

The blood volume of normal dogs. Blood volume figures for twenty-two normal dogs, young and adult, maintained on the usual mixed diet are given in table 5. The average for all animals shows 10.13 cc. of blood per 100 grams of body weight. This value corresponds fairly closely to the results of Keith, Rowntree and Geraghty (dye method) (2), and to those of Meek and Gasser (acacia method) (5), but is considerably higher than that obtained by Grehand and Quinquaud (6) with the carbon monoxide method (8.2 cc. per 100 grams body weight), and also that obtained by the method originally employed by Welcker, which depends on washing out or extracting all of the blood and estimating the hemoglobin. By this method about 7.8 cc. of blood per 100 grams body weight was obtained (7). The reasons for the difference between these methods are as yet not entirely clear. This problem is receiving further study in this laboratory.

TABLE 6

Female mongrel bull dog, weight 22 pounds. Hemorrhage 262 cc. in 3 minutes

DOG NUMBER	DATE	BLOOD VOLUME	PLASMA VOLUME	TOTAL CELL VOLUME	HEMATOCRIT (CELLS)	TOTAL PROTEIN	ALBUMIN	GLOBULIN	NON-PROTEIN
					per cent				
16–160	March 4	1006	533	473	47.0				
	March 6 (before hemorrhage)					5.7	4.1	1.6	2.5
	March 6 (1 minute after hemorrhage)					5.6	4.1	1.5	2.5
	March 6 (5 minutes after hemorrhage)	741	363	378	51.0	5.3	4.1	1.2	2.3
	March 6 (30 minutes after hemorrhage)				47.0	5.2	4.1	1.1	2.1
	March 7					4.7	3.5	1.2	2.3
	March 9	844	591	253	30.0	4.3	3.3	1.0	2.5
	March 15					4.9	3.7	1.2	2.5

In this series practically no difference exists in the amount of blood per unit body weight in respect to the size of the animal.

Hemorrhage experiments. In a number of animals the effect of acute hemorrhage on blood and plasma volume was studied. The protocols of four such experiments are presented in tables 6, 7, 8 and 9. In all experiments healthy, sound young dogs were used. The first blood volume estimation in each experiment was performed two days

before the day of the hemorrhage. It is recognized that in individual cases the blood volume may change somewhat in the space of two days. In order to minimize this error the animals were kept under as nearly constant conditions as possible throughout the course of the experiment, all animals being kept on a uniform diet of cracker-meal, lard and butter for a period of five days preceding the hemorrhage. Hemorrhage was effected by rapid withdrawal of blood by aspiration through a needle inserted in the external jugular vein. The animals were but slightly depressed and showed but a slight temporary amount of dyspnea as a result of the loss of blood.

TABLE 7

Black male mongrel pup, weight 20 pounds. Hemorrhage 404 cc. in 3 minutes

DOG NUMBER	DATE	BLOOD VOLUME	PLASMA VOLUME	TOTAL CELL VOLUME	HEMATOCRIT CELLS	TOTAL PROTEIN	ALBUMIN	GLOBULIN	NON-PROTEIN
					per cent				
16–140	March 4	1616	711	905	56	5.0			
	March 6 (before hemorrhage)					5.4	3.7	1.7	2.5
	March 6 (1 minute after hemorrhage)					5.2	4.1	1.1	2.1
	March 6 (5 minutes after hemorrhage)	1130	531	599	53	5.3	3.9	1.4	2.1
	March 6 (30 minutes after hemorrhage)					5.1	3.7	1.4	2.1
	March 7					4.9	3.6	1.3	2.2
	March 9	1222	880	342		4.5	2.9	1.6	2.2
	March 15					4.7	3.1	1.6	2.2

The effect of the procedure on the blood volume, plasma volume and cell volume is summarized in table 10. The amount of plasma and of cells removed is calculated from the total amount of blood withdrawn on the assumption that the blood withdrawn contained the same percentage of cells as was present in the blood at the time of performing the first blood volume. Although a certain amount of variation exists in individual experiments, the averages show that the fall in both cell volume and in plasma volume is within a few cubic centimeters of the theoretical. In the course of three days, however, the plasma volume has returned to a level slightly above the original level. Due to the decreased cell volume the blood volume is still

TABLE 8

Female bull coach mongrel, weight 18 pounds. Hemorrhage 244 cc. in 4 minutes

DOG NUMBER	DATE	BLOOD VOLUME	PLASMA VOLUME	TOTAL CELL VOLUME	HEMATOCRIT (CELLS)	TOTAL PROTEIN	ALBUMIN	GLOBULIN	NON-PROTEIN
					per cent				
17–157	March 4	976	537	439	45				
	March 6 (before hemorrhage)					5.7	4.3	1.4	2.1
	March 6 (1 minute after hemorrhage)					5.4	3.7	1.7	2.1
	March 6 (5 minutes after hemorrhage)	724	427	297	41	5.0	3.5	1.5	1.8
	March 6 (30 minutes after hemorrhage)				40	4.9	3.5	1.4	1.8
	March 7					5.0	3.9	1.1	1.6
	March 9	818	597	221	27	4.9	3.9	1.0	1.8
	March 15					4.9	3.5	1.4	2.1

TABLE 9

Male mongrel bull dog, weight 27.5 pounds. Hemorrhage 264 cc. in 2 minutes

DOG NUMBER	DATE	BLOOD VOLUME	PLASMA VOLUME	TOTAL CELL VOLUME	HEMATOCRIT (CELLS)	TOTAL PROTEIN	ALBUMIN	GLOBULIN	NON-PROTEIN
					per cent				
17–205	March 4	1055	538	517	49				
	March 6 (before hemorrhage)					3.4	3.9	1.5	2.7
	March 6 (1 minute after hemorrhage)					5.8	4.2	1.6	2.1
	March 6 (5 minutes after hemorrhage)	757	424	333	44	5.3	4.1	1.2	2.3
	March 6 (30 minutes after hemorrhage)					5.2	3.9	1.3	2.1
	March 7					5.2	3.5	1.7	2.3
	March 9	840	605	235	28	4.9	3.5	1.2	2.5
	March 15					5.0	3.9	1.1	2.1

below the original figure. The fact that the cell volume is lower at the end of three days than immediately following the hemorrhage is a peculiar and as yet unexplained phenomenon. More data are being collected on this point.

In all of the hemorrhage experiments the serum proteins were estimated by the micro-refractometric method of Robertson (8). A summary of the figures given in detail in the protocols of the individual experiments is presented in table 11. The amount of circulating serum proteins is decreased as a result of hemorrhage by very nearly the theoretical amount. Regeneration, however, has in the course of

TABLE 10

The effect of hemorrhage upon blood volume

DOG NUMBER	WITHDRAWN		BLOOD VOLUME			PLASMA VOLUME			TOTAL CELL VOLUME		
	Of plasma	Of blood cells	Before	Five minutes after	Three days after	Before	Five minutes after	Three days after	Before	Five minutes after	Three days after
	cc.	*cc.*									
16–160	139	123	1006	741	844	533	363	591	475	378	253
16–140	142	262	1616	1130	1222	711	531	880	905	599	342
17–157	134	110	976	724	818	537	427	597	439	297	221
17–205	135	129	1055	757	840	538	424	605	517	333	235
Average.....	138	156	1164	838	931	580	436	668	583	402	263

TABLE 11

The effect of hemorrhage upon the circulating serum proteins

DOG NUMBER	SERUM PROTEIN WITHDRAWN	CIRCULATING SERUM PROTEINS			SERUM PROTEIN REGENERATED	
		Before hemorrhage	Five minutes after hemorrhage	Three days after hemorrhage	In five minutes	In three days
	grams	*grams*	*grams*	*grams*	*grams*	*grams*
16–160	8.07	30.38	19.24	25.41	−3.07	3.10
16–140	9.74	38.39	28.14	41.36	−0.51	12.71
17–157	7.72	30.61	21.35	29.25	−1.54	6.36
17–205	7.91	29.05	22.47	29.65	+1.33	8.51
Average.....	8.36	32.11	22.80	31.42	−0.95	7.67

three days restored the circulating proteins almost to the original figure. It is noteworthy, however, that the concentration of protein in the serum is still considerably below the original.

In conclusion and on the behalf of the Department of Anatomy and the Hooper Foundation we wish to express our appreciation for invaluable assistance given by Dr. Carl Alsberg, Chief of the Bureau

of Chemistry, Department of Agriculture, Washington, and Dr. H. D. Gibbs, Chemist in charge of the Color Laboratory, Bureau of Chemistry, Department of Agriculture, Washington. These gentlemen supplied practically all the dye used in the first two papers of this series.

SUMMARY

A simple and accurate method of blood volume determination is outlined in detail. Suitable controls of many factors are submitted and the method tested *in vitro*.

The average normal blood volume for young active dogs of medium weight as determined by this method may be stated as 10.1 per cent body weight. Individual differences are considerable (table 5).

After the rapid withdrawal (hemorrhage) of a known amount of blood, this blood volume method performed immediately will show a *calculated blood volume* remaining which is very close to the *estimated blood volume*.

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BLOOD VOLUME STUDIES

II. Repeated Determination of Blood Volume at Short Intervals by Means of the Dye Method

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A simple method for the estimation of blood volume in the same animal at short intervals is much to be desired. An accurate method for the estimation of blood volume repeatedly at intervals of minutes or hours will at once give data of interest and value. Such data are not at present available. With the ability to estimate blood volume changes during short intervals we are at once able to study the exchange of fluids which may go on in acute shock, or after hemorrhage or induced plethora. Under such conditions one suspects that there may be exchange of fluid between the tissues and the circulating blood but there are no published experiments which are not open to criticism.

Certain observations of this nature have been reported by a few of the earlier workers—Keith, Rowntree and Geraghty (1), Robertson and Bock (2) and Lamson (3), but no details are given as to the exact method used and only one or two observations are recorded in each report. For this reason and because of our earlier experiments we conclude that these observations were not entirely satisfactory.

When one attempts to repeat the blood volume estimation on the same animal at short intervals he is at once confronted by the fact that after one such determination the plasma remains colored for a period varying from several hours to several days. The presence of dye in the plasma complicates all subsequent blood volume determinations. In order to determine the blood volume a second time under such circumstances, one may again inject intravenously the customary amount of dye and estimate colorimetrically the *increase* in dye concentration in the plasma as a result of such injection. Theoretically this increase in color intensity might be estimated by subtracting the colorimetric reading of a sample of plasma drawn before injection of the

dye from the colorimetric reading of another sample taken after such injection. The increase in color intensity thus determined is, however, subject to the error of two colorimetric readings, the sum of which may in some cases be considerable. For example, the second determination may be made after an interval of a few hours. At such time the amount of dye in the plasma may be small and therefore difficult to estimate accurately because of the normal yellow plasma tint. The procedure used to minimize this error is given below. The mathematical soundness of this procedure will be shown.

METHOD

For a repeated blood volume estimation the dye is injected and the samples collected in the same way as was outlined in the preceding paper (4). Part of this may for convenience be repeated. A hypodermic needle is inserted into the jugular vein and 10 cc. of blood drawn (with as little compression of the vein as possible) into a dry well-vaselined syringe. This blood is immediately run into a 15 cc. graduated hematocrit tube containing 2 cc. of a 1.6 per cent solution of sodium oxalate. The blood and oxalate are mixed by inversion and the tube stoppered. An amount of brilliant vital red (1 per cent) equal in cubic centimeters to the weight of the animal in pounds divided by 11 (i.e., 1 cc. per 5 kilos body weight) is drawn up into a syringe along with 5 to 10 cc. of 0.9 per cent saline. This dye solution is now injected into the jugular vein, the dye being washed completely out of the syringe into the blood stream by means of a few cubic centimeters of normal saline. Four minutes after the injection of dye a clean needle is again inserted into the jugular vein (preferably of the opposite side) and another 10 cc. sample of blood is drawn and placed in another 15 cc. hematocrit tube also containing 2 cc. of 1.6 per cent sodium oxalate.

The second blood volume determination may be done at such time as may be desired—for example, after a period of 10 or 20 minutes. The procedure is exactly similar to that already described and the time interval of 4 minutes is the same as before, that is, 4 minutes from time of injection of the second dye sample to the time of removal of the second dye plasma sample.

The hematocrit tubes are now centrifugalized at 2500 revolutions a minute for 30 minutes. The total contents of the tube and the number of cubic centimeters of blood cells are now noted. Colorimetric readngs are then made and the plasma volume estimated as follows:

Calculation of the second blood volume

Let D = the number of cubic centimeters of 1 per cent dye injected into the animal.

C_1 = the total number of cubic centimeters of oxalated plasma present in the first sample of blood drawn minus 2, divided by the total number of cubic centimeters of oxalated plasma present in the same tube. This value expresses the ratio of the actual concentration of dye in the plasma when diluted with oxalate solution, to the value when not so diluted.

C_2 = the total number of cubic centimeters of oxalated plasma present in the second sample of blood drawn minus 2, divided by the total number of cubic centimeters of oxalated plasma present in the same tube. The significance of this figure is the same as that described for C_1 above.

R_1 = the observed colorimetric reading of a sample of dye-tinged plasma (diluted with two parts of saline) taken immediately before the injection of the dye for the second blood volume estimation read against a standard prepared as follows: Of a dye solution containing 0.75 cc. of 1 per cent dye diluted to 200 cc. with water take 5 cc. To this amount add 5 cc. of 0.9 per cent saline and 5 cc. of normal dye-free plasma.

R_2 = the observed colorimetric reading (expressed in per cent) of a sample of dye-colored plasma (diluted with two parts of saline) taken 4 minutes after the injection of the dye for the repeated blood volume estimation, against a standard prepared as follows: 5 cc. of a dye solution containing 0.75 cc. of 1 per cent dye in 200 cc. of water, + 5 cc. of 0.9 per cent saline + 5 cc. of the dye-tinged plasma taken immediately before the injection of dye for the repeated blood volume estimation.

Then:

$$\text{The plasma volume} = \frac{2666666\, D\, C_1 C_2}{C_1 R_2 (R_1 + 100) - 100\, C_2 R_1}$$

$$\text{And the blood volume} = \frac{\text{Plasma Volume} \times 100}{\text{Per Cent Plasma}}$$

The total blood-cell volume = blood volume minus plasma volume.

The method above described is essentially the same method as is employed in the estimation of the first blood volume. It will be recalled that in the method as outlined in the previous paper the standard against which the unknown (diluted with two parts of saline) was read, was made up of equal parts of saline, standard dye solution (0.75 cc.

of 1 per cent dye diluted with water to 200 cc.) and normal plasma taken from the same animal immediately before injection of the dye. In performing the second blood volume the unknown and standard are prepared in exactly the same way. In this case, however, the plasma taken immediately before the injection of dye is itself dye-colored (from dye remaining from the previous blood volume determination). This amount of dye (R_1) is present in both standard and unknown. Hence: $\frac{R_1+R_3}{R_1+100} = \frac{R_2}{100}$ whence R_1 = the colorimetric reading of the plasma before the injection of dye against a standard prepared in the customary way for the first blood volume estimation (equal parts of normal plasma, saline and standard dye solution). R_3 represents the increase in concentration of dye in the plasma as a result of the injection of dye. This formula simply means that the unknown ($R_1 + R_3$) divided by the standard ($R_1 + 100$) equals the observed colorimetric reading expressed in parts per 100 $\left(\text{i.e. } \frac{R_2}{100}\right)$.

The soundness of this last formula is illustrated experimentally in experiment no. 243. In this experiment the standard employed in no. 4 may be taken as 100. The standard used in no. 5 then equals $100 + R_1$ where R_1 (according to no. 4) equals 75. The unknown in no. 5 also contains R_1 and hence may be represented as $R_1 + R_3$ where R_3 equals the increase in dye concentration to be determined. R_2, the observed reading, is 86. By substituting in the formula $\frac{R_1+R_3}{R_1+100} = \frac{R_2}{100}$ one obtains $\frac{75+R_3}{75+100} = \frac{86}{100}$ Solving, $R_3 = 75.5$.

It can readily be seen from no. 3 that the increase (R_3) in dye concentration over the residual is 75. The increase as determined colorimetrically with the use of the formula differs from the correct value, therefore, by only 0.5 per cent.

Experiment 243. Measurement of the color increase resulting from the addition of dye to dye-tinged water.

1. Seventy-five one hundredths cubic centimeter of 2 per cent brilliant vital red are accurately diluted with water to 200 cc. in a volumetric flask.

2. Six hundred and seventy-five thousandths cubic centimeter of a 2 per cent solution of the same dye is similarly diluted with water to 240 cc. This is equivalent to diluting 0.56 cc. of dye to 200 cc.

3. Fifty-six hundredths cubic centimeter more of 2 per cent dye is accurately diluted with no. 2 to 200 cc. in a volumetric flask.

4. A standard is prepared as follows: 5 cc. of no. 1 + 10 cc. of water. Against this standard 5 cc. of no. 2 diluted with 10 cc. of water reads 75 per cent.

5. A second standard is prepared as follows: 5 cc. of no. 1 + 5 cc. of no. 2 + 5 cc. of water. Against this standard 5 cc. of no. 3 diluted with 10 cc. of water reads 86 per cent.

It was stated above that mathematically it would be possible to determine the increase in color intensity by a simple subtraction of the residual dye concentration (R_1) from the total dye concentration after addition of more dye. With such procedure it was pointed out that the error in the estimated increase is the algebraic sum of the errors of these two colorimetric readings. Computation shows that by the procedure outlined with the use of the above formula the error in the estimated increase (R_3) is not so great as this algebraic sum provided that R_1 is less than R_3. Moreover as R_1 becomes smaller its accurate determination becomes increasingly difficult particularly since the interference due to the normal pigments of the plasma becomes more marked. Fortunately an error of considerable magnitude may be made in the estimation of R_1 without seriously affecting the value of R_3. For example if through error in colorimetric reading R_1 in experiment no. 243 had been incorrectly read as 70 instead of 75, R_3 would have been calculated to be 76.2 instead of 75.5—an error of less than 1 per cent.

In all blood volume determinations the plasma is diluted by the oxalate solution which must necessarily be added to prevent coagulation. Correction must therefore be made for such dilution. The formula $\frac{R_1 + R_3}{R_1 + 100} = \frac{R_2}{100}$ becomes

$$\frac{\frac{R_1C_2}{C_1} - R_3}{R_1 + 100} = \frac{R_2}{100} \text{ or } R_3 = \frac{R_1R_2C_1 + 100\ C_1R_2 - 100\ R_1C_2}{100\ C_1}$$

In the previous paper it was found that Plasma Volume = $\frac{26666.666\ D\ C_2}{R}$. Since R in this formula corresponds to the R_3 in the formula derived above (R_3 being the increase in dye concentration without correction for dilution by oxalate), R_3 may be substituted in the formula for plasma volume with the following result:

$$\text{Plasma Volume } \frac{2666667\ D\ C_1C_2}{C_1R_2(R_1 + 100) - 100\ C_2R_1}$$

The experiment cited below (from exper. 218) illustrates the procedure.

Dog, weight 47.5 pounds. At 10.45 a.m. a simple blood volume estimation was carried out. Preparations were then made to repeat the determination as has been outlined above. At 11.07 a.m. a sample of blood drawn from the jugular vein was placed in a 15 cc. hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate. After centrifugalization this tube was found to contain 5.95 cc. of blood cells. Its total content was 14.75 cc. C_1 estimated as described above is $\frac{68}{88}$. At 11.08 a.m. brilliant vital red (4.30 cc. of 1 per cent solution) together with about 6 cc. of 0.9 per cent sodium chloride were injected into the jugular vein. At 11.12 a.m. another sample of blood was drawn into another hematocrit tube containing also 2 cc. of 1.6 per cent sodium oxalate. After centrifugalization this tube was found to contain 5.6 cc. of blood cells. Its total content was 14.6 cc. C_2 therefore is $\frac{70}{90}$.

One cubic centimeter of plasma from the blood sample drawn at 11.07 a.m. was mixed in a small tube with 2 cc. of 0.9 per cent sodium chloride. This solution was read against a standard prepared as follows: 5 cc. of a dye solution containing 0.75 cc. of 1 per cent brilliant vital red diluted to 200 cc. with water + 5 cc. of 0.9 per cent sodium chloride + 5 cc. of normal plasma (obtained from the same animal at 10.43 a.m., i.e., before he had received the first injection of dye). The test solution reads against this standard 76 per cent. R_1 therefore = 76.

A second standard, this time containing dye-tinged plasma, was prepared as follows: 5 cc. of plasma from the sample of blood drawn at 11.07 a.m. + 5 cc. of a dye solution containing 0.75 cc. of 1 per cent brilliant vital red diluted to 200 cc. with water + 5 cc. of 0.9 per cent sodium chloride. A test solution was prepared by diluting 1 cc. of plasma obtained from the sample of blood drawn at 11.12 a.m. with 2 cc. of 0.9 per cent saline. This solution against the above standard reads 90 per cent. R_2 therefore = 90.

Substituting these values in the formula:

$$\text{Plasma Volume} = \frac{2666667\ D\ C_1C_2}{C_1R_2(R_1 + 100) - 100\ C_2R_1}$$

we have

$$\text{Plasma Volume} = \frac{(2666667)\ (4.30)\left(\frac{68}{88}\right)\left(\frac{70}{90}\right)}{\left(\frac{68}{88}\right)(90)\ (76 + 100) - (100)\left(\frac{70}{90}\right)(76)} = 1089 \text{ cc.}$$

The per cent plasma (obtained from an average of the two hematocrit tubes) is 54.4. Since the blood volume $= \frac{\text{Plasma Volume} \times 100}{\text{Plasma Per Cent}}$ the blood volume $= \frac{1089 \times 100}{54.4} = 2002.$

By difference the total cell volume is 913 cc.

DISCUSSION

In tables 1 and 2 the dye method for the repeated estimation of blood volume is tested out *in vitro* on large quantities of oxalated dog's blood. In the experiment cited in table 1 three separate blood volume determinations are made on known amounts of the same sample of oxalated blood. In no case was the error greater than 3.1 per cent while the average error for the three determinations was 2.5 per cent. In the experiment cited in table 2 the errors in the estimation by the dye method were 2.5 and 5.0 per cent respectively in each of two determinations The mechanical errors involved in the repeated estimations of blood volume by the dye method under such experimental conditions do not exceed 5 per cent.

In the three experiments, tables 3, 4 and 5, repeated blood volume determinations have been made on the same dogs at short intervals of time. In all cases repeated determinations on the same animal give similar results. In no case does any blood volume determination differ from the average of all determinations made on the same animal by more than 5.0 per cent.

We wish to emphasize, however, that these figures apply to determinations completed within two to three hour periods. When longer intervals elapse between blood volumes we may observe fluctuations in blood cell and plasma figures which we cannot at present explain. We feel that sufficient data are submitted to show that these fluctuations are not due to errors inherent in this proposed method. When we consider the various physiological reactions of the blood vessels in response to familiar digestive stimuli we need not be surprised at fluctuations in circulating blood volume. These physiological reactions are merely suggested as a type of the many changes which might modify the fluid or cells circulation in the body at any given time.

Experiment 268. The repeated blood volume method carried out in vitro on oxalated blood.

1. Seven hundred and fifty cubic centimeters of blood were drawn from the jugular veins of each of four normal dogs into each of two bottles containing 75 cc. each of 1.6 per cent sodium oxalate. This blood was thoroughly mixed. A graduated 15 cc. hematocrit tube was filled.

2. Nine hundred cubic centimeters of no. 1 were poured into a dry 1000 cc. volumetric flask. Two cubic centimeters of 1 per cent brilliant vital red were added and the flask made up to mark with no. 1. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time 14 cc. were quickly pipetted into a dry 15 cc. graduated hematocrit tube.

3. Five hundred cubic centimeters of no. 2 were poured into a volumetric flask calibrated to hold 510 cc. One and two hundredths cubic centimeter of 1 per cent brilliant vital red were added and the flask made up to mark with no. 2. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time a dry graduated 15 cc. hematocrit tube was filled with the mixture.

4. Four hundred and eighty-five cubic centimeters of no. 3 were poured into a volumetric flask calibrated to hold 490 cc. Ninety-eight hundredths cubic centimeter of a 1 per cent brilliant vital red were added and the flask made up to mark with no. 3. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time a dry graduated 15 cc. hematocrit tube was filled with the mixture.

All of the hematocrit tubes were corked and centrifugalized for 30 minutes at 2500 revolutions a minute. The quantity of packed cells and of supernatant fluid in each tube was noted.

The standard solutions and dilutions employed in obtaining the colorimetric readings given below were made in the usual manner except that the aqueous dye solution from which the standards were prepared contained 1 cc. of dye diluted to 200 cc. with water instead of the customary 0.75 dye diluted to 200 cc. with water.

In the first volume estimation (no. 2 above) $R_2 = 88$ per cent. R_1 of course in this case is zero.

In the second volume estimation (no. 3 above) $R_2 = 91$ per cent. From the preceding paragraph it is obvious that $R_1 = 88$ per cent.

In the third volume estimation (no. 4 above) R_2 (no. 4 above) = 94 per cent. R_1 (estimated from the sample prepared in no. 3 above) is 170 per cent.

Since the hematocrit tubes receiving the blood contained no oxalate solution, C_1 and C_2 in all cases equal unity. The final results must be multiplied by $\frac{3}{4}$ because of the stronger standard used.

The results of the three volume estimations are given in the table below.

Experiment 253. The repeated blood volume method carried out in vitro on oxalated blood.

1. Three hundred cubic centimeters of blood were drawn from a normal dog into a flask containing 35 cc. of 1.6 per cent sodium oxalate. This blood was thoroughly mixed. A graduated 15 cc. hematocrit tube was filled.

2. Two hundred cubic centimeters of no. 1 were poured into a dry 250 cc. volumetric flask. Then 0.455 cc. of 1 per cent brilliant vital red was added and the flask made up to mark with no. 1. The contents of the flask were thoroughly mixed by rotation and inversion for 5 minutes. At the end of this time 14 cc. were quickly pipetted into a dry 15 cc. hematocrit tube.

3. One hundred and fifty cubic centimeters of no. 2 were then poured into a volumetric flask calibrated to hold 200 cc. Then 0.356 cc. of 1 per cent brilliant vital red was added and the flask made up to mark with no. 2. The contents of the flask were thoroughly mixed by rotation and inversion for five minutes. At the end of this time a dry graduated 15 cc. hematocrit tube was filled with the mixture. All of the hematocrit tubes were corked and centrifugalized for 30 minutes at 2500 revolutions a minute. The quantity of packed cells and of supernatant fluid in each tube was noted.

TABLE 1

Experiment 268. The repeated blood volume method carried out in vitro on oxalated blood

VOLUME DETERMINATION	VOLUME OF OXALATED PLASMA		HEMATOCRIT (CELLS)	VOLUME OF OXALATED BLOOD			TOTAL VOLUME OF PACKED CELLS	
	Actual (by hematocrit)	Estimated by dye method		Actual	Estimated by dye method	Error	Actual (by hematocrit)	Estimated by dye method
	cc.	*cc.*	*per cent*	*cc.*	*cc.*	*per cent*	*cc.*	*cc.*
First	465	454	53.5	1000	976	2.4	535	522
Second	271	246	53.2	510	526	3.1	266	280
Third	261	234	53.2	490	500	2.0	261	266

The standard solutions and dilutions employed in obtaining the colorimetri readings given below were made in the usual manner.

In the first volume estimation (in no. 2 above) $R_2 = 100$. R_1 of course in this case is zero.

In the second volume estimation (no. 3 above) $R_2 = 100$. From the preceding paragraph it is obvious that $R_1 = 100$ per cent.

Since the hematocrit tubes receiving the blood contained no oxalate solution, C_1 and C_2 in all cases equal unity.

The results of the two volume estimations are given in table 2 below.

TABLE 2

Experiment 253. The repeated blood volume method carried out in vitro on oxalated blood

VOLUME DETERMINATION	VOLUME OF OXALATED PLASMA		HEMATOCRIT (CELLS)	VOLUME OF OXALATED BLOOD			TOTAL VOLUME OF PACKED CELLS	
	Actual (by hematocrit)	Estimated by dye method		Actual	Estimated by dye method	Error	Actual (by hematocrit)	Estimated by dye method
	cc.	*cc.*	*per cent*	*cc.*	*cc.*	*per cent*	*cc.*	*cc.*
First	115	121	53.9	250	262	5.0	135	141
Second	92	95	53.9	200	206	3.0	108	111

Experiment 218. Repeated determination of blood volume on the same animal. Dog 19–117. Short haired, adult male bull dog. Good condition. Weight 47.5 pounds. Blood volume determinations were carried out according to the procedure already described and at the intervals indicated below:

First blood volume determination made at 10.43 a.m.

Second blood volume determination made at 11.08 a.m.

Third blood volume determination made at 11.27 a.m.

Due to the taking of blood samples for other purposes a larger amount of blood was withdrawn than is customary—a total of 108 cc.

The results of the determinations are given in table 3 below.

TABLE 3

Experiment 218. Repeated determination of blood volume on the same animal

VOLUME DETERMINATION	TIME	PLASMA VOLUME	HEMATOCRIT (CELLS)	TOTAL BLOOD VOLUME	TOTAL VOLUME OF CELLS
		cc.	*per cent*	*cc.*	*cc.*
First.....	10.43	1048	47.5	1994	946
Second.....	11.08	1074	45.6	1972	898
Third.....	11.27	1088	44.1	1946	858
Average.....		1070	45.7	1971	901

Experiment 227. Repeated determination of blood volume on the same animal. Dog 17–160. Short haired, brown adult male mongrel. Good condition. Weight 45 pounds. Blood volume determinations were carried out according to the procedure already described and at the intervals indicated below:

First blood volume determination made at 9.58 a.m.
Second blood volume determination made at 10.15 a.m.
The total amount removed was 45 cc.
The results of the determinations are given in table 4 below

TABLE 4

Experiment 227. Repeated determination of blood volume on the same animal

VOLUME DETERMINATION	TIME	PLASMA VOLUME	HEMATOCRIT (CELLS)	TOTAL BLOOD VOLUME	TOTAL VOLUME OF CELLS
		cc.	*per cent*	*cc.*	*cc.*
First.....	9.58	1478	56.7	3413	1935
Second.....	10.15	1478	57.7	3494	2016
Average.....		1478	57.2	3454	1976

Experiment 246. Repeated determination of blood volume on the same animal. Dog 19–111. Long haired, adult male shepherd. Good condition. Weight 35 pounds. Blood volume determinations were carried out according to the procedure already described and at the intervals indicated below:

First blood volume determination made at 10.30 a.m.
Second blood volume determination made at 10.45 a.m.
Third blood volume determination made at 1.55 p.m.
Total amount of blood removed was 69 cc.
The results of the determinations are given in table 5 below.

In conclusion I wish to express appreciation to Dr. G. H. Whipple and to Mr. A. E. Belt for advice given throughout the course of this work and for valuable assistance given in the matter of arrangement and presentation of the subject matter contained in this paper.

TABLE 5

Experiment 246. Repeated determination of blood volume on the same animal

VOLUME DETERMINATION	TIME	PLASMA VOLUME	HEMATOCRIT (CELLS)	TOTAL BLOOD VOLUME	TOTAL VOLUME OF CELLS
		cc.	*per cent*	*cc.*	*cc.*
First	10.30	909	48.5	1765	856
Second	10.45	896	47.1	1694	798
Third	1.55	994	45.4	1821	827
Average		933	47.0	1760	827

SUMMARY

A method is outlined in which the dye blood volume method is adapted to repeated determinations on the same animal at short intervals. The soundness of the method is demonstrated by controls done *in vitro* as well as repeated determinations performed in rapid succession on the same animal.

The experimental error does not exceed 5 per cent. Fluctuations in blood volume greater than this are sometimes seen over long periods of time. These fluctuations arise from physiological factors, the exact nature of which is not yet understood.

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BLOOD VOLUME STUDIES

III. Behavior of Large Series of Dyes Introduced into the Circulating Blood

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Blood volume determinations by means of dye substances injected intravenously are now familiar procedures. A dye suitable for this work must be non-toxic and not permanently stored by the body cells. More important, the dye must remain in the circulating plasma at least for several minutes after its injection before its removal from the plasma is much in evidence. "Vital red" dyes have been used for blood volume determinations in human beings and animals with no untoward effects. Some of the errors which may creep into these determinations have been pointed out in other papers of this series (papers I and II). The method used in these experiments is described in detail below.

In view of the increasing interest and importance of blood volume studies it seemed desirable to study with care many dye subtances which might be of value in future work—possibly of greater value than the "vital red" series. This study has been extended to include a number of dyes which are excreted in considerable measure by the kidneys or liver. In many instances the behavior of a certain dye was well known and is included in our tables mainly for comparison and control of other factors. Other dyes were tested because of a suggestive chemical constitution which would seem to indicate a possible physiological relationship to other familiar dyes. Still other dyes of this series were employed because they had been studied as to other aspects of their behavior in living animals by workers in the Department of Anatomy. It need not be repeated that at present *the behavior of a given dye in the blood stream cannot be predicted from any analysis of its chemical formula.*

We have tested by the method described below a series of over sixty dye substances. The dyes are predominantly of the benzidine series, are non-toxic and not chemically changed in the blood plasma. For the sake of convenience in tabulation these dyes have been grouped into three main divisions but it should be kept in mind that there is no sharp line of demarkation. These groups fade into each other. Group I contains dyes resembling the "vital red" series which persist in the blood plasma. Group II contains dyes which leave the blood stream rapidly and appear in the urine, for example, phenolsulphonephthalein. Group II contains dyes which leave the blood stream with considerable rapidity but do not appear to any degree in the urine. What becomes of such substances we are unable to say but it is evident that these dyes are changed by contact with the living cells so that they are no longer recognizable.

From the standpoint of blood volume the dyes of group I are of especial interest, but some significance attaches to the physiological behavior of the dyes in groups II and III. An analysis of the dyes in group I (table 5) will show a blue azo dye (T-1824) which we believe to be slightly superior to the best of the vital red series. This dye gives a blue color to the plasma which is very easy to read and can be colorimetrically determined with great accuracy. It leaves the blood plasma very slowly and hemolysis which may be present as a confusing factor in certain experiments is more easily recognized than is the case with the red dyes. Six other dye substances (table 5) may be classified with the "vital red" group for routine blood volume work and if available may be substituted for vital red.

METHOD

Unless otherwise noted a 1 per cent solution of the dye made up in distilled water and filtered is used for injection. Calculated on the basis of 2 mgm. per kilogram of body weight, the proper amount of dye is slowly injected into the jugular vein from a glass syringe. This syringe is then rinsed by withdrawing a little blood which is reinjected and followed by a little saline. Previous to the dye injection, a 10 cc. sample of blood is withdrawn in a clean 10 cc. syringe (rinsed in 0.9 per cent salt solution) and emptied into a 15 cc. graduated hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate solution. The tube is inverted twice to insure thorough mixing. Following this two 10 cc. samples of blood are withdrawn and emptied into two vaselined test

tubes. This provides serum for the standard dye mixture. To determine the rapidity of disappearance of the dye from the blood stream, samples are taken in vaselined test tubes after the injection of the dye at the following intervals and in the amounts indicated: after 2, 6, 10, 25 minutes, one 10 cc. sample; after 4, 15 and 40 minutes, two 10 cc. samples. After stoppering, the tubes are centrifuged at 2500 revolutions per minute for 30 minutes. The hematocrit readings (total volume minus 2 cc. oxalate, total cell volume and volume of red cells) are recorded. The normal serum for the standard is pipetted off into a clean dry test tube. The dyed serum samples are also pipetted off into a series of clean, dry tubes.

Standard: One-half cubic centimeter of the 1 per cent dye solution (5 mgm. dye) is made up to 100 cc. with distilled water in a volumetric flask.

Five cubic centimeters of the above dye standard are diluted with 5 cc. normal serum and 5 cc. 0.9 per cent salt solution (dilution 1 in 3). This standard reads 100 per cent.

Dilution: Tubes—Dyed plasma mixed with 0.9 per cent salt solution in the proportion 2 cc. to 4 cc. respectively (dilution 1 in 3).

Colorimetric determinations: Each of the samples of the diluted dyed serum placed successively in a colorimeter is compared with the standard in the wedge of the colorimeter and the average of five readings is recorded as the color per cent of the sample.

Computation of results: From the hematocrit readings per cent plasma and red cells may be easily obtained. It will be recalled that each 40 cc. of the standard contains 2 mgm. of the dye.

$$\text{Plasma Volume in cc.} = \frac{\text{Weight in Kilograms} \times 40}{\text{Average Colorimetric Reading}} \times 100$$

$$\text{Blood Volume in cc.} = \frac{\text{Plasma Volume}}{\text{Plasma Per Cent}} \times 100$$

Determination of amount of dye in the urine: The dog is catheterized and the bladder washed out with about 20 cc. of warm water at the beginning of the experiment. Collection of urine by catheter is made one hour after injection of the dye and at hourly intervals thereafter until the amount of dye present is too small for colorimetric determination.

Total amount of urine plus washings from each collection is measured, neutralized and made up to a volume with sufficient water to give a

color slightly less intense than the standard dye solution with which a filtered sample is compared. The volume may be 100 cc. or some multiple of 100.

Standard: One-half cubic centimeter of a 1 per cent solution of the dye, made up to 100 cc. volume with distilled water or with diluted neutral urine when specimens are not the same color as the standard aqueous solution. This standard contains 10 mgm. of dye per cubic centimeter. Whenever the first hour sample of urine apparently contains no dye, or only a trace, a portion is made acid and another alkaline to make sure that the dye is not present in some colorless form or compound easily dissociated by acid or alkali.

Dogs: For the major part of the work six normal male dogs were selected ranging in weight from 45 to 63 pounds. They were used in sequence each day so that each dog was used but once a week. This arrangement gives time for the dye to leave the plasma between determinations and also prevents the development of anemia from too frequent hemorrhage, as each experiment necessitates the withdrawal of 130 cc. of blood. The dogs are always fed after the experiment. This insures an absence of lipemia which may be a disturbing factor.

Controls for the method: For each dye duplicate experiments have been made on different dogs and with many of the dyes three to six determinations were made. Small dogs were used in addition to the large ones giving results which are consistent with those obtained for the standard dogs. Duplicate tests have been made with certain dyes on the same dog with essentially identical results.

Duplicating the blood samples for the 4, 15 and 40 minute periods gives control for the technique employed including the colorimetric readings. As a rule both tubes taken at the same time interval gave identical readings, the exception being a variation of not more than 2 per cent. To obviate the personal factor in making colorimetric readings, all determinations were made by the same observer throughout this series of experiments.

Often the samples and standard have been kept in the ice-box for a second colorimetric comparison on the following day. These late readings were practically identical with those of the freshly prepared specimens. All recorded observations, however, were made on the same day of the experiment.

Experiments using large amounts of dyes: By injecting 20 mgm. per kilogram of body weight, curves showing the rate of disappearance of dyes from the plasma have been worked out for brilliant vital red

(new); T-1824; T-1835 (alkaline); trypan blue (dyes slowly removed from the blood stream); phenolsulphonephthalein; phenoltetrachlorphthalein; crystal S. scarlet and Buffalo fast crimson (dyes rapidly removed from the blood stream). (Refer to table 11.)

The measured amount of dye (20 mgm. per kilo body weight) is slowly injected and two 10 cc. samples of blood withdrawn at 4 and 40 minutes after injection and one 10 cc. sample 1, 2, 3, 4, 6, 8 and 12 hours after injection if the dye still persists in the plasma. After 12 hours, samples are taken on successive days at approximately the same hour until the amount of dye in the plasma is too small for satisfactory colorimetric determination. Dilution of the plasma is necessary for the first specimens which are all read against the usual standard prepared as described above.

In the following list of dye substances three main groups are made as follows:

Group I. Dyes behaving in the blood stream like brilliant vital red (blood volume group).

Group II. Dyes which show rapid disappearance from the blood plasma and are excreted in large measure by the kidneys. (Type of phenolsulphonephthalein).

Group III. Dyes rapidly lost from the plasma yet not excreted by the kidneys. Certain dyes of this group show a substantial trace in the urine. (Intermediate group.)

LIST OF DYE SUBSTANCES

Group I. Blood volume group

Designation	*Chemical constitution*
Brilliant vital red	Orthotolidin + 1 mol. β naphthylamine 3.6 disulphonic acid and 1 mol. β naphthylamine 6 monosulphonic acid.
Vital new red (E and S)	A tetrazo dye belonging to a group made by combining urea derivatives with amido naphthols, naphthols and naphthylamines.
Vital new orange (E and S)	A tetrazo dye belonging to a group made by combining urea derivatives with amido naphthols, naphthols and naphthylamines.
No. 176	Tetrazo dye formed by combining para para diamido diphenyl ureas with various naphthols, amido naphthols and naphthylamines.
Trypan red	Benzidine monosulphonic acid combined with 2 mol. β naphthylamine 3.6 disulphonic acid.

Dianil granat B	Benzidine combined with 1 mol. 2 amido-8 naphthol 6 monosulphonic acid and 1 mol. β naphthylamine 3.6 disulphonic acid.
No. 86	Ben idine combined with 1 mol. β naphthylamine 3.6 disulphonic acid and 1 mol. β naphthylamine 6 monosulphonic acid.
Columbia blue R	Benzidine combined with 1 mol. α naphthol 3.8 disulphonic acid and 1 mol. 1.8 amido naphthol 4 disulphonic acid.
No. 276	Benzidine combined with 1 mol. H acid and 1 mol. 2.8 ethyl amido naphthol 6 monosulphonic acid.
Direkt himmelbau gruenlich	Dianisidine combined with 2 mol. 1.8 amido naphthol 2.5 disulphonic acid.
Chicago blue 4B	Dianisidine combined with 1 mol. 1.8 amido naphthol 2.4 disulphonic acid and 1 mol. 1.8 amido naphthol 4 monosulphonic acid.
Chicago blue 6B	Dianisidine combined with 2 mol. 1.8 amido naphthol 2.4 disulphonic acid.
No. 2826 B	Dianisidine combined with 1.8 amido naphthol 2.4 disulphonic acid.
Hoechst No. 9	Ortho tolidin combined with 2 mol. β naphthylamine 6 monosulphonic acid.
No. 204	Ortho tolidin combined with 1 mol. β naphthylamine and 1 mol. β naphthylamine 6 monosulphonic acid.
T-148	Ortho tolidin + 2 mol. α naphthylamine 4.8 disulphonic acid.
O-Tolidin + 1 NH_2 (4.8)	Ortho tolidin + 2 mol. α naphthylamine 4.8 disulphonic acid.
Brilliant purpurine R	Ortho tolidin combined with 1 mol. α naphthylamine 4 monosulphonic acid and 1 mol. β naphthylamine 3.6 disulphonic acid.
T + 2 mol. 1 naph. 3.6 disulf	Ortho tolidin + 2 mol. α naphthylamine 3.6 disulphonic acid.
T + 2 mol. β naph. 5.7 disulf	Ortho tolidin + 2 mol. β naphthylamine 5.7 disulphonic acid.
T-1824	Ortho tolidin combined with 2 mol. 1.8 amido 2.4 disulphonic acid.
No. 2826 A	Ortho tolidin combined with 2 mol. 1.8 amido 2.4 disulphonic acid.
2 mol. 1824 − 1 mol. 0-tolidin	Ortho tolidin combined with 2 mol. 1.8 amido naphthol 2.4 disulphonic acid.
Dianil blue 2 R	Ortho tolidin combined with 1 mol. chromatrope acid and 1 mol. α naphthol 4 monosulphonic acid.
T + 1835 (alkaline)	Ortho tolidin combined with 2 mol. 1.8 amido naphthol 3.5 disulphonic acid in alkaline solution.

T + 2 mol. 1846.....Ortho tolidin combined with 2 mol. 1.8 amido naphthol 4.6 disulphonic acid.

Columbia blue G.....Ortho tolidin combined with 1 mol. α naphthol 3.6 disulphonic acid and 1 mol. 1.8 amido naphthol 4 monosulphonic acid.

Wasserblau.....Sulphonic acid of triphenyl para rosaniline.

Indazarun B. B.....Dianisidine combined with 1 mol. 1.7 dioxy-2 naphtholic acid-4 sulphonic acid and 1 mol. β naphthol 3.6 disulphonic acid.

No. 173.....Tetrazo dye formed by combining para para diamido diphenyl ureas with various naphthols, amido naphthols and naphthylamines.

Group II. Renal excretion group

Phenolsulphonephthalein

Buffalo fast crimson.....Mono azo dye formed by linkage of acetyl β phenylendiamine combined with α naphthol 3.6 disulphonic acid.

Crystal s. scarlet.....Mono azo dye formed by combining α naphthylamine with β naphthol 6.8 disulphonic acid.

Tolan red.....Mono azo dye formed by combining aniline with 1.8 amido naphthol 4.6 disulphonic acid.

No. 226.....Benzidine disulphonic acid with 2 mol. 1.8 amido naphthol 4.6 disulphonic acid.

No. 227.....Benzidine disulphonic acid with 2 mol. 2.8 amido naphthol 6 monosulphonic acid.

No. 228.....Benzidine disulphonic acid with 2 mol. 2.5 naphthol 7 monosulphonic acid.

T-1835.....Ortho tolidin combined with 2 mol. 1.8 amido 3.5 disulphonic acid (probably in acid solution).

No. 225.....Benzidine meta disulphonic acid + 2 mol. H acid.

No. 105.....Benzidine combined with 2 mol. chromatrope acid.

L. T. 297.....Ortho tolidin disulphonic acid combined with 2 mol. amido naphthol 3.6 disulphonic acid.

T. disulfosaure + H acid.....Ortho tolidin disulphonic acid combined with 2 mol. amido naphthol 3.6 disulphonic acid.

Alizarin green S.....A mixture of tri and tetra oxy anthraquinone quinolines and their sulphonic acid derivatives.

No. 155.....Para para diamido stilbene combined with 2 mol. H acid.

Indigo disulfosaure.....Indigo disulphonic acid.

Group III. Intermediate group

New Bordeaux L	Benzidine combined with 2 mol. β naphthol 8 monosulphonic acid.
Baumwoll rubin	Benzidine combined with 1 mol. α naphthylamine 4 monosulphonic acid and 1 mol. β naphthol 8 monosulphonic acid.
Naphthamine black C. E	Benzidine combined with 1 mol. of 2.8 amido naphthol 6 monosulphonic acid and 1 mol. H acid.
Naphthamine black R. E	Benzidine combined with 1 mol. 2 amido 8 naphthol 6 monosulphonic acid + 1 mol. of the K acid.
Trisulfon violet	Benzidine combined with 1 mol. β naphthol and 1 mol. α naphthol 3.6.8. trisulphonic acid.
No. 114	Benzidine combined with 1 mol. H acid and 1 mol. α naphthol 4 monosulphonic acid.
No. 316	Benzidine combined with 1 mol. H acid and 1 mol. 2.8 ethyl amido naphthol 6 monosulphonic acid.
No. 221	Dianisidine combined with 1 mol. Neville-Winther acid and 1 mol. 1.7 dioxy 2 naphthoe 4 sulphonic acid.
No 295	Dianisidine combined with 1 mol. α naphthol 4 monosulphonic acid and 1 mol. 1.7 dioxy 2 naphthoe 4 disulphonic acid.
No. 481 Mulheim blue II	Ortho tolidin combined with 2 mol. chromotrope acid.
Lichtgrün S. F	Triphenyl methane dye. Chlor methylate of hexa methyl para rosaniline chloride.
Trypan blue	Benzidine dye: Ortho tolidin combined with 2 mol. 1.8 amido naphthol 3.6 disulphonic acid.
Congo blue (X pure)	Ortho tolidin combined with 1 mol. α naphthol 4 monosulphonic acid and 1 mol. H acid.
No. 222	Dianisidine combined with 1 mol. H acid and 1 mol. 1.7 dioxy 2 naphthoeic acid 4 sulphonic acid.
No 181	Tetrazo dye formed by combining para para diamido diphenyl ureas with various naphthols, amido naphthols and naphthylamines.
No. 230	Benzidine disulphonic acid combined with 2 mol. β naphthylamine 7 monosulphonic acid.
No. 246	Tetrazo dye formed by combining para para diamido diphenyl ureas with various naphthols, amido naphthols and naphthylamines.

EXPERIMENTAL OBSERVATIONS

It will be seen from table 1 that the different vital red dyes show considerable differences in the color intensities of the standard 1 per cent solution. Whether this may be due in part to inert salts present in the dye powder is not determined. It will be seen that the dyes may differ slightly in their physiological reaction in the plasma (table 3). For example, brilliant vital red II leaves the circulation with slightly greater speed than some of the other vital red dyes.

TABLE 1

Comparison of vital red dyes

DESIGNATION	CHEMICAL CONSTITUTION	COLOR STRENGTH
		per cent
Brilliant vital red (new)	Ortho tolidin combined with one molecule β naphthylamine 3.6 disulphonic acid and one molecule β naphthylamine 6 monosulphonic acid	100
Brilliant vital red (old)	Ortho tolidin combined with one molecule β naphthylamine 3.6 disulphonic acid and one molecule β naphthylamine 6 monosulphonic acid	75
Brilliant vital red II	Ortho tolidin combined with one molecule β naphthylamine 3.6 disulphonic acid and one molecule β naphthylamine 6 monosulphonic acid	50
Original Rowntree dye	Diamino dixylyl methane combined with two molecules β naphthol 3.6 disulphonic acid	60
Rowntree II no. 273	Dichlor benzidine combined with 2 molecules β naphthylamine 3.6 disulphonic acid	100

Standardization of the six large dogs by means of these vital red dyes is detailed in table 2. It will be noted that these standard observations were made at intervals during the period in which these dogs were used to standardize the other dyes as indicated in other tables to follow. Table 2 may be used as a reference table to show the average blood volume figures as well as maximal and minimal readings for blood volume in individual dogs as determined by the vital red dye group. These same standardized dogs are used throughout the entire series of observations tabulated below. It is interesting to note the average figure for blood per kilo equals 93 and average cell hematocrit equals 50.4 per cent. It

may be stated again that these dogs were all strong, well-nourished, rather inactive adult males.

The speed with which these vital red dyes are removed from the circulating plasma can be estimated from the color readings given in table 3. During a period of 40 minutes on the average the color readings fall 13 per cent—that is, from an average reading of 90 color per cent to an average of 77 color per cent. There is good evidence that

TABLE 2

Blood volume determinations with vital red dyes

DATE	DYE	DOG	WEIGHT	RED CELLS	PLASMA	COLOR (4 MIN.)	PLASMA VOLUME	BLOOD VOLUME	BLOOD PER KILOGRAM
			kgm.	*per cent*	*per cent*	*per cent*	*cc.*	*cc.*	
11/18	Brilliant vital red (new)	16–178	27.04	43.2	55.1	89	1215	2206	82
11/26	Brilliant vital red (old)	16–178	27.10	46.4	53.1	99	1095	2063	76
2/27	Brilliant vital red II	16–178	28.45	46.6	52.4	94	1210	2309	81
12/15	Original Rowntree dye	16–178	26.95	44.0	54.5	99	1088	1996	74
12/30	Brilliant vital red (new)	18–60	21.90	48.6	50.4	83	1055	2093	91
11/22	Brilliant vital red (old)	18–60	22.30	51.2	47.7	95	939	1968	88
11/29	Original Rowntree dye	18–60	21.80	52.7	46.3	82	1063	2295	105
2/25	Original Rowntree dye	18–60	21.90	47.5	51.5	76	1152	2236	102
11/21	Brilliant vital red (new)	19–63	21.60	50.0	48.8	80	1080	2213	102
11/30	Brilliant vital red (old)	19–63	21.90	53.6	45.4	87	1006	2215	101
12/28	Original Rowntree dye	19–63	23.00	50.2	48.6	80	1152	2370	102
11/23	Brilliant vital red (new)	19–70	21.30	52.8	46.1	94	906	1965	92
4/29	Brilliant vital red II	19–70	26.80	45.8	53.7	99	1083	2017	75
5/6	Brilliant vital red (new)	17–154	26.90	54.4	45.1	96	1123	2490	92
4/30	Brilliant vital red II	17–154	27.00	53.8	45.2	95	1137	2515	93
3/4	Brilliant vital red (new)	19–71	24.10	53.4	46.1	96	1004	2193	91
1/22	Brilliant vital red II	19–71	23.50	54.8	45.2	86	1093	2418	103
2/11	Rowntree II no. 273	19–71	23.50	51.4	47.6	81	1160	2437	104
3/31	Rowntree II no. 273	19–71	25.50	51.7	47.2	88	1160	2457	96
Average			24.4	50.4	48.6	89			93

the dye is thoroughly mixed with the circulating blood at the end of 2 minutes as these readings are so constant when compared with the 4-minute samples which average 1 color per cent less. All the blood volume determinations are figured from the 4-minute reading which is arbitrarily taken as the optimum figure.

The data on color concentration in blood plasma at varying time intervals are presented in table 3 but the same data are given in different

TABLE 3

Color determinations vital red dyes readings in per cent

DYE (DESIGNATION)		BRILLIANT VITAL RED (NEW)						BRILLIANT VITAL RED (OLD)		BRILLIANT VITAL RED II				ORIGINAL ROWNTREE DYE			ROWNTREE DYE II	
Dog number		16-178	18-60	19-63	19-70	17-154	19-71	18-60	19-63	16-178	19-70	17-174	19-71	18-60	18-60	19-63	19-71	19-71
Weight, pounds		59.5	48	47.5	47.0	59	53	49.13	48.25	62.5	59	59.5	51.5	48.0	48.13	50.75	51.75	56.0
Time after injection readings in per cent	2 min.	90	85	88	95	97	97	99	90	94	100	95	88	85	77	80	81	90
	4 min.	89	83	86	94	96	96	95	87	94	99	95	86	82	76	80	81	88
	6 min.	88	83	84	94	95	96	88	86	93	97	93	85	82	75	79	81	87
	10 min.	84	83	80	94	93	95	86	83	91	95	88	83	81	73	77	80	85
	15 min.	83	82	77	90	90	93	85	80	88	92	85	81	80	71	75	78	83
	25 min.	81	80	73	86	88	91	83	78	81	87	81	74	77	70	71	76	80
	40 min.	79	79	70	79	87	89	79	76	76	83	77	70	73	68	68	75	77
Total loss in color per cent—40 minutes		11	6	18	16	10	8	20	14	18	17	18	18	12	9	12	6	13

form in table 4 to show the loss in color per cent reading during the time intervals as tabulated. There is practically always a slight fall in color readings in the interval between the 2- and 4-minute samples (table 4—first column, 2 to 4)—average 1.4 color per cent. The same fall is noted in the interval between the 4- and 6-minute samples—average 1.2 color per cent. The loss of dye from the plasma is less

TABLE 4

Decrease in color concentration of plasma; minutes after injection

DYE (DESIGNATION)	DOG	2 TO 4 MINUTES	4 TO 6 MINUTES	6 TO 10 MINUTES	10 TO 15 MINUTES	15 TO 25 MINUTES	25 TO 40 MINUTES	TOTAL 40 MINUTES COLOR PER CENT
Brilliant vital red (new)	18-60	2	0	0	1	2	1	6
	19-63	2	2	4	3	4	3	18
	16-178	1	1	4	1	2	2	11
	19-70	1	0	0	4	4	7	16
	17-154	1	1	2	3	2	1	10
	19-71	1	0	1	2	2	2	8
Brilliant vital red (old)	18-60	4	7	2	1	2	4	20
	19-63	3	1	3	3	2	2	14
Brilliant vital red II	16-178	0	1	2	3	7	5	18
	19-70	1	2	2	3	5	4	17
	17-154	0	2	5	3	4	4	18
	19-71	2	1	2	2	7	4	18
Original Rowntree	18-60	3	0	1	1	3	4	12
	18-60	1	1	2	2	1	2	9
	19-63	0	1	2	2	4	3	12
Rowntree II no. 273	19-71	0	0	1	2	2	1	6
	19-71	2	1	2	2	3	3	13
Average		1.4	1.2	2.1	2.2	3.3	3.1	13 3

rapid as we approach the 40-minute period—for example, a loss of 3.1 color per cent during 15 minutes preceding the 40-minute reading. During the first 10 minutes after the dye injection there is an average loss in color per cent reading of 1 per cent per 2-minute intervals. It will be noted that brilliant vital red (old) and (II) leave the blood stream a little more rapidly on the average than do the other dyes in this group. A part of this difference may be apparent and not real.

TABLE 5

Color determinations for dyes of group I A: readings in per cent

DYE	T-1824			TWO MOLECULES 1824 ONE MOLECULE O-TOLIDIN		No. 2826A		T-1835 (ALKALINE)			CHICAGO BLUE 6B		DIANIL BLUE 2R		No. 2826B		HOECHST 9		No. 173	
Dog number	18-60	19-63	17-154	19-63	19-71	17-154	16-178	17-154	18-60	19-63	18-60	19-71	19-70	19-71	19-70	17-154	16-178	19-70	19-71	18-60
Weight in pounds	48	49.25	61-0	49-25	55.0	60.5	60.5	59.63	47.38	52.63	47.0	49.5	50.25	51.0	47.5	58.75	63.13	45.63	56.5	51.5
Time after injection reading in per cent: 2 min.	78	83	98	90	97	104	106	90	76	76	76	97	92	84	97	110	87	74	98	85
4 min.	78	83	98	90	96	102	104	89	75	75	75	97	91	83	96	110	87	73	97	84
6 min.	78	82	96	89	95	100	102	89	74	74	75	96	90	82	95	108	87	73	96	82
10 min.	77	80	95	87	93	99	100	87	74	73	74	95	88	81	88	106	85	71	94	81
15 min.	77	79	95	83	91	97	99	86	72	71	72	95	86	80	88	106	82	67	90	80
25 min.		75	93	78	88	97	99	84	70	68	71	94	84	75	84	106	75	58	88	77
40 min.	70	74	91	74	85	95	98	83	69	64	68	93	81	70	83	104	71	54	83	72
Total loss in color per cent in 40 minutes	8	9	7	16	12	9	8	7	7	12	8	4	11	14	14	6	16	20	15	13

These two dyes are paler than the others and the color readings therefore a little less accurate. The differences, however, are too small to have much significance.

In group I–A are included seven dyes which compare favorably with the vital red series and may be substituted if occasion arises. There are five blue dyes in this group and the blue color in itself has certain advantages over the red dye colors. Many workers can estimate blue colors more accurately, more rapidly and with less fatigue than is the case with red colors. Slight hemolysis can be recognized more easily when the blue dyes are used. Even a trace of hemolysis will rarely confuse one who is thoroughly familiar with the blood volume work no matter what dye is being used.

One blue dye is particularly well adapted to the routine blood volume work and is preferred by us when compared with the vital red group. The difference, however, is not great but for many workers the blue dye has advantages. This azo dye is tested in table 5 in seven different experiments. Three different samples of the same dye are used (T-1824 and 2-mol. 1824, 1 mol O-tolidin and No. 2826 A) and tabulated in the first three groups of table 5. It will be noted that this blue dye is slow to leave the circulating blood plasma, if anything slightly more so than the vital red series.

The second blue dye (T-1835 alkaline) is also slow to leave the blood stream during the observed period of 40 minutes but the three color readings are somewhat lower than the usual average. We are not prepared to explain this point but many more observations must be made before any such unusual reaction can be accepted.

AVERAGE COLOR CONCENTRATION	VITAL RED	T-1824	T-1835 (ALKALINE)
Two-minute samples	90	94	81
Four-minute samples	88	93	80
Forty minute samples	78	84	72

The above tabulation shows that the vital red figures compare closely with the blue azo dye T-1824. The readings of T-1835 (alkaline) are low but these figures represent the average of too few observations while the vital red figures represent a great many experiments.

It will be seen that the curve of dye removal from the blood stream is the same for this group of dyes as for the vital red group. If anything these dyes (group 1-A) are even more slowly removed from the blood stream, particularly the blue dyes.

The two red dyes (Hoechst 9 and No. 173) of group 1-A behave very much like the vital red group. Too few observations have been made to determine this point beyond question.

TABLE 6

Decrease in color concentration of plasma for dyes of group I-A. Minutes after injection

DYE	DOG	2 TO 4 MINUTES	4 TO 6 MINUTES	6 TO 10 MINUTES	10 TO 15 MINUTES	15 TO 25 MINUTES	25 TO 40 MINUTES	TOTAL 40 MINUTES COLOR PER CENT
T-1824	18–60	0	0	1	0		7	8
	19–63	0	1	2	1	4	1	9
	17–154	0	2	1	0	2	2	7
2 mol. 1824, 1 mol. 0-tolidin	19–63	0	1	2	4	5	4	16
	19–71	1	1	2	2	3	3	12
No. 2826 A	17–154	2	2	1	2	2	2	9
	16–178	2	2	2	1	0	1	8
T-1835 (alkaline)	17–154	1	0	2	1	2	1	7
	18–60	1	1	0	2	2	1	7
	19–63	1	1	1	2	3	4	12
Chicago blue 6B	18–60	1	0	1	2	1	3	8
	19–71	0	1	1	0	1	1	4
Dianil blue 2R	19–71	1	1	1	1	5	5	14
	19–70	1	1	2	2	2	3	11
No. 2826 B	19–70	1	1	7	0	4	1	14
	17–154	0	2	2	0	0	2	6
Hoechst 9	16–178	0	0	2	3	7	4	16
	19–70	1	0	2	4	9	4	20
No. 173	19–71	1	1	2	4	2	5	15
	18–60	1	2	1	1	3	5	13

The corresponding blood volumes on the standardized dogs are figured in table 7 and are found to be in harmony with observations made with vital red dyes in table 2. The first experiment in table 7 gives a figure of 70 cc. per kilo for the blood volume which is open to

criticism. This figure is very low as compared with many others on this same dog and is much below the average. It is more than probable that some technical error crept into this experiment—possibly some dye contaminating a container, syringe or hypodermic needle.

Group I-B contains many dyes which are fairly satisfactory in many respects when used for blood volume determinations. These dyes do not leave the blood stream rapidly and in only one or two instances

TABLE 7

Blood volume determinations group I-A

DATE	E	DOG	WEIGHT	RED CELLS	PLASMA	COLOR PER 4 CENT MINUTES	PLASMA VOLUME	BLOOD VOLUME	BLOOD PER KILOGRAM	DECREASE IN COLOR CONCENTRATION 40 MINUTES
			kgm.	*per cent*	*per cent*		*cc.*	*cc.*		
1/15	No. 2826A	16-178	27.5	44.2	54.8	104	1058	1930	70	9
1/2	Hoechst No. 9	16-178	28.2	48.1	50.9	87	1298	2550	90	16
12/9	T + 1824	18-60	21.8	50.2	48.8	78	1120	2295	105	8
2/3	T + 1835 (alkaline)	18-60	21.5	49.5	49.5	75	1149	2321	107	7
1/7	Chicago 6 B	18-60	21.3	49.5	50.0	75	1138	2276	106	8
6/30	No. 173	18-60	23.4	48.4	50.1	84	1116	2227	95	13
12/7	T + 1824	19-63	22.4	52.6	45.8	83	1079	2356	104	9
1/22	2 mol. 1824 + 1 mol. 0-tolidin	19-63	22.4	54.0	45.0	90	995	2211	98	16
3/1	T + 1835 (alkaline)	19-63	23.7	51.0	48.0	75	1266	2637	111	12
2/5	Dianil blue 2 R	19-70	23.7	46.1	52.9	91	1044	1973	83	11
1/11	No. 2826 B	19-70	21.6	43.7	55.3	96	900	1627	96	14
1/4	Hoechst No. 9	19-70	20.2	45.8	53.2	73	1109	2084	102	20
3/3	T +1824	17-154	27.7	54.5	44.5	98	1132	2544	92	7
1/10	No. 2826 A	17-154	27.5	54.6	44.4	102	1078	2428	88	9
2/1	T +1835 (alkaline)	17-154	27.1	60.5	39.5	89	1195	3026	111	7
1/16	No. 2826 B	17-154	26.7	62.5	36.5	110	970	2657	99	6
5/2	2 mol. 1824 + 1 mol. 0-tolidin	19-71	25.0	53.8	45.7	96	1042	2280	91	12
1/14	Chicago blue 6 B	19-71	22.5	55.6	43.4	97	928	2138	95	4
2/4	Dianil blue 2 R	19-71	23.2	55.2	44.3	83	1118	2523	109	14
6/10	No. 173	19-71	25.7	57.2	41.8	97	1059	2533	99	15

are moderately toxic. As a group the colors are pale and correspondingly difficult to read. A pale color cannot be as accurately determined and work with such dyes is more time consuming. It should be stated, however, that trypan red, indazarun B. B. and no. 204 when injected in the strength of 4 mgm. per kilo do give satisfactory colors for routine work. Used in this strength these three dyes compare favorably with the vital red group.

TABLE 8

Blood volume determinations, group I-B

DATE	DYE	DOG	WEIGHT	RED CELLS	PLASMA	COLOR PER 4 CENT MINUTES	PLASMA VOLUME	BLOOD VOLUME	BLOOD PER KILOGRAM	DECREASE IN COLOR CONCENTRATION 2-40 MINUTES
			kgm.	*per cent*	*per cent*		*cc.*	*cc.*		
10/14	Vital new red	16–178	28.0	45.5	53.4	104	1077	2016	72	21
1/8	Chicago blue 4B	16–178	28.0	48.8	50.2	104	1077	2145	76	15
1/23	No. 204	16–178	28.5	45.5	53.5	98	1163	2173	76	22
1/30	Indazarun B.B.	16–178	28.2	50.9	49.0	97	1163	2373	84	11
1/17	Vital new red	18–60	21.2	49.5	50.0	87	977	1940	91	8
5/15	No. 176	18–60	24 8	46.3	52.7	91	1090	2068	82	10
4/26	No. 86	18–60	24.5	45.3	53.7	82	1195	2225	90	10
3/5	No. 204	18–60	22.1	49.0	50.5	79	1119	2215	100	18
3/22	T − 148	18–60	23.0	47.1	51.9	93	989	1905	83	8
12/16	T + 2 mol. β naphth. 5.7 disulph.	18–60	21.5	50.2	48.7	85	1011	2076	96	13
2/21	Trypan red	19–63	23.0	50.3	48.7	73	1263	2593	112	12
12/14	Direkt himmel blau gruenlich	19–63	23.0	50.7	47.9	79	1167	2436	105	13
1/13	Chicago blue 4 B	19–63	22.4	55.8	43.2	87	1030	2384	106	8
12/21	T + 2 mol. 1846	19–63	23.8	49.5	49.5	72	1325	2676	113	8
1/31	Indazarun B.B.	19–63	21.7	54.1	44.9	77	1114	2481	114	14
6/27	No. 176	19–70	25.7	57.0	41.9	114	901	2150	83	14
2/19	Trypan red	19–70	23.6	42.2	56.8	93	1017	1790	75	8
12/24	T + 2 mol. 1 naph. 3.6 disulf.	19-70	21.3	35.3	63.7	83	1265	1985	93	23
12/17	T + 2 mol. 1 naph. 3.6 disulf.	19 70	21.7	44.8	54.2	92	945	1743	80	9
	No. 86	17–154	27.5	53.2	45.8	95	1157	2526	92	7
6/28	No. 276	17–154	27.7	58.6	40.4	99	1122	2777	100	21
12/13	Direkt himmelblau gruenlich	17–154	27.7	55.5	43.4	97	1144	2635	94	5
12/20	2 mol. β naph. 5.7 disulfosaure	17–154	27.7	55.7	43.7	102	1088	2489	89	14
4/16	Wasserblau	17–154	27.0	53.2	46.2	85	1165	2502	92	8
5/16	No. 276	19–71	25.2	52.2	47.2	90	1122	2377	94	18
3/25	T − 148	19–71	25.0	52.8	46.7	92	1087	2327	93	7
12/18	T + 2 mol. 1846	19–71	23.6	51.1	48.3	79	1197	2478	104	5
4/15	Wasserblau	19–71	25.3	53.8	45.7	100	1014	2219	87	13

Wasserblau is decolorized by the blood plasma but the color returns on the addition of 2 drops of concentrated hydrochloric acid to blood serum tubes and standard. Brilliant purpurine in the strength of 4

mgm. per kilo will cause hemolysis in the circulating blood, and some clinical evidence of intoxication.

Vital new red, vital new orange, direkt himmelblau gruenlich and T-148 persist in the blood stream for 7 to 10 days or longer. This is an objection to the routine use of these four dyes and may introduce difficulties when a series of observations is to be made upon the same animal.

As regards the dyes in group I-B, too few observations have been made to settle all points of interest which concern blood volume work. Some single observations are not even included in table 8 as they show nothing unusual and the dyes gave no promise of being useful in this work. This statement applies to columbia blue R, O-tolidin+1 NH_2 (4.8), dianil granat B, brilliant purpurine R, columbia blue G, and some others listed but not specifically mentioned.

TABLE 9

Summary. Dyes of group I

	VITAL RED DYES 19 EXPERIMENTS			OTHER DYES OF GROUP I-A 20 EXPERIMENTS			DYES IN GROUP I-B 28 EXPERIMENTS		
	Average	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum
Kilogram weight	24.4	21.3	28.45	24.1	21.3	28.25	24.7	21.2	28.5
Red cells per cent	50.4	43.2	54.8	51.8	43.7	62.5	50.1	35.3	58.6
Color per cent (4-minute sample)	89	76	99	89	73	110	90.3	72	114
Blood per kilogram body weight	93	74	105	92	70	111	92	72	114
Decrease in color per cent 2-40 minutes	13	6	20	11	6	20	12	5	23

Dyes of group II

This group includes the dyes which are eliminated more or less completely by the kidneys. Phenolsulphonephthalein is a dye familiar to everyone and we record in table 10 familiar data concerning renal excretion of this dye. The amounts injected are somewhat larger than the usual clinical dose but the per cent excretion is normal for dogs. The rapid disappearance from the blood stream is well shown. Much of the phthalein has left the blood by the end of 2 minutes and almost all of it by the end of the next 4 minutes. Phenoltetrachlorphthalein (1) which is known to be excreted in the bile is taken out of

the blood under similar conditions with even greater speed. There is practically complete removal of this dye from the blood serum by the end of 2 minutes following its injection. Perhaps the relatively greater size of the liver and consequent larger minute circulation volume as compared with the kidneys may account for this difference.

Several of the dyes listed in table 11 (group II) are rapidly removed from the blood stream but none with the speed just recorded for phenolsulphonephthalein. Buffalo fast crimson, crystal S. scarlet, tolan red, T-1835 and indigo disulfosaure are dyes which show minimal color readings in the plasma 6 minutes after the dye injection. Probably the dye is almost completely removed within a period of 15 minutes.

TABLE 10

*Decrease in color concentration of plasma and excretion by the kidneys—Phenolsulphonephthalein**

DATE	DOG	WEIGHT	COLOR PER CENT 2 MINUTE SAMPLE	LOWEST COLOR PER CENT RECORDED	TIME INTERVAL OF LOWEST COLOR PER CENT	TOTAL DYE RECOVERED IN URINE	TIME RECOVERY OF DYE IN URINE
		kilograms			*minutes*	*per cent*	*hours*
5/7	16–178	24.65	26	18	4	62.8	4
5/8	18–60	24.5	38	27	4	72.7	4
5/7	19–63	22.8	28	20	4	60.5	6
12/10	19–70	21.3	41	28	4	60.0	2
12/6	17–154	27.0	50	38	4	70.3	3
5/8	19–71	25.5	35	23	4	64.9	4
Average		24.3	36	26		65.2	

* 1.3 mgm. per kilo body weight.

Buffalo fast crimson seems to be more like phenolsulphonephthalein in that over 50 per cent of the dye is excreted in the urine. It seems that this dye should be more carefully studied as to its excretion by the kidney in health and disease. The other dyes show wide variations in the amount recovered from the urine after intravenous injection. It is possible that conditions present in the kidney, bladder or urine may modify the yield to a considerable extent. A more careful study of this group of dyes should be undertaken and may yield information of value concerning the secretory activity of normal and abnormal cells as concerns these particular dyes. It will be of some interest to determine whether the same or different limitations apply to all these dyes as is true for phenolsulphonephthalein.

The other dyes in group II leave the blood stream less rapidly and in most instances a readable amount of dye remains at the end of 40

TABLE II

Dyes of group II

DATE	DYE	DOG	WEIGHT	COLOR PER CENT 2 MINUTES	LOWEST COLOR PER CENT RECORDED	TIME INTERVAL OF LOWEST COLOR PER CENT	TOTAL DYE RECOVERED IN URINE	URINE COLLECTION
			kgm.			*minutes*	*per cent*	*hours*
12/12	T + 1835 (B 1841)	16–178	27.0	48	33	6	21.1	4
12/14	L. T. 297	16–178	27.75	78	54	40	14.4	3
11/1	Alizarin green	16–178	26.76	61	39	45	24.8	3
3/6	No. 227	16–178	29.0	65	36	40	8.1	6
5/23	Buffalo fast crimson	18–60	25.0	61	35	15	53.0	3
11/13	Crystal s. scarlet	18–60	22.3	63	44	6	20.6	2
4/12	Indigo disulfosäure	18–60	23.8	42	20	6	53.4	3
12/23	L. T. 297	18–60	22.2	77	54	40	15.2	4
4/19	No. 225	18–60	24.5	65	36	40	17.2	4
1/2	No. 228	18–60	21.3	73	48	40	8.8	4
6/7	Crystal s. scarlet	19–63	23.6	66	30	15	5.3	1
4/4	Indigo disulfosäure	19–63	23.5	55	35	6	22.3	4
1/6	No. 105	19–63	23.2	69	44	40	4.0	2
4/25	No. 225	19–63	24.0	55	23	40	14.3	4
3/4	No. 227	19–63	21.9	65	36	40	11.7	6
12/2	T − disulfosäure + H. acid	19–70	20.3	74	47	40	6.6	4
1/29	No. 226	19–70	21.9	72	37	40	20.7	6
11/27	T − 1835	17–154	27.1	50	37	6	15.4	4
1/3	No. 105	17–154	27.2	86	60	40	18.2	2
1/25	No. 228	17–154	26.9	75	55	40	6.2	3
12/3	Buffalo fast crimson	19–71	22.9	58	28	15	51.1	3
1/7	Tolan red 4 B	19–71	23.3	59	31	10	29.2	4
12/31	Tolan red 4 B	19–71	23.3	52	30	10	29.5	4
3/21	T + 2 mol. 1.8 amido naph. 3.5 disulfosäure	19–71	25.0	53	28	6	7.5	4
12/11	T + 1835 (B. 1841)	19–71	23.3	50	35	4	13.7	4
11/25	T + 1835	19–71	24.3	44	32	6	7.2	4
11/15	Alizarin green	19–71	23.4				16.0	1
1/28	No. 226	19–71	23.3	73	41	40	19.1	5

minutes. One is not surprised therefore to note that the dye elimination in the urine is scanty and rarely exceeds 15 to 20 per cent.

Group III-A

This group contains a number of dyes which exhibit peculiar reactions in the blood stream when compared with the dyes in group I. At present we have insufficient experimental data to give the correct explanation so that discussion of various possibilities will be brief.

Two dyes (new Bordeaux L and lichtgrün S. F.) leave the blood stream with considerable rapidity yet do not appear in the urine (table 12). We have not been able to test for elimination in the bile so this cannot be excluded. Lichtgrün S. F. was decolorized in the blood plasma. Two drops of acetic acid brought back the color but hydrochloric acid did not develop the color (compare wasserblau). New Bordeaux L. was tested *in vitro* against normal serum which did not influence the dye even after three days' incubation at 38°C. It is possible, of course, that the endothelial cells of the living body are concerned in this reaction.

TABLE 12

Dyes of group III-A

DATE	DYE	DOG	WEIGHT	COLOR CONCENTRATION 2 MINUTES	LOWEST COLOR PER CENT RECORDED	TIME INTERVAL FOR LOWEST COLOR PER CENT
			kgm.	*per cent*		*minutes*
3/26	New Bordeaux L	16–178	27.0	62	35	10
3/20	Baumwoll rubin	16–178	27.0	86	48	40
3/13	Naphthamine black R. E.	16–178	28.3	77	41	40
3/19	New Bordeaux L	18–60	22.3	70	37	10
2/17	Naphthamine black R. E.	18–60	21.95	55	44	40
4/5	No. 413 trisulfon violet	18–60	24.5	50	40	40
4/11	Lichtgrün S. F.	19–63	24.0	75	43	10
4/18	No. 114	19–63	24.0	94	66	40
6/24	No. 316	19–63	24.1	68	34	40
6/30	No. 295	19–63	24.5	75	52	40
4/3	Mulheim blue	19–70	26.5	99	69	40
4/10	No. 413 trisulfon violet	19–70	26.7	55	33	40
4/17	No. 114	19–70	27.0	100	73	40
3/24	Baumwoll rubin	17–154	27.0	87	55	40
4/9	Mulheim blue	17–154	27.6	110	85	40
2/15	No. 221	17–154	27.5	99	70	40
3/10	No. 316	17–154	27.0	72	38	40
6/9	No. 295	17–154	27.5	95	71	40
4/8	Lichtgrün S. F.	19–71	25.25	79	39	10
3/	No. 221	19–71	24.0	94	55	25
2/17	Naphthamine black R. E.	19–71	24.0	82	54	40

Other dyes in table 12 show occasionally a figure for the 40-minute period indicating a slow removal but a low initial reading. This, however, is not constant and the low initial reading is perhaps an accident due to unknown factors. There is a possibility of dye removal by the process of coagulation—that is, the dye may be carried out of solution by the clot. Unfortunately this point has not been controlled in this group as has been done in the vital red group. When these experiments were begun this possibility was not considered and oxalate plasma was not used in place of coagulated serum. In view of the irregular figures noted in table 12 for the same dye we must consider this possibility until it is disproved.

TABLE 13

Decrease in color concentration of plasma. Group III-B.
Minutes after injection

DATE	DYE	DOG	2 TO 4 MINUTES	4 TO 6 MINUTES	6 TO 10 MINUTES	10 TO 15 MINUTES	15 TO 25 MINUTES	25 TO 40 MINUTES	TOTAL 40 MINUTES COLOR PER CENT
2/6	Congo blue B	16–178	2	0	2	3	13	5	25
2/20	No. 230	16–178	1	3	6	3	9	6	28
3/11	No. 230	18–60	4	3	4	3	4	6	24
6/25	No. 181	18–60	2	4	3	5	5	13	32
5/3	Trypan blue	19–63	3	3	6	3	3	6	24
2/7	Congo blue B	19–63	1	3	2	2	6	5	19
5/14	No. 246	19–63	4	3	7	5	5	5	29
5/12	No. 181	19–70	0	8	3	4	20	6	41
2/26	Trypan blue	19–71	0	1	2	3	4	12	22
6/24	No. 246	19–71	1	3	5	8	8	5	30

The dyes given in table 13 all give a rather low initial reading as well as a pretty rapid removal during the 40-minute period. It is possible at least that the endothelial cells are concerned in a part of this reaction. It should be noted that the urine usually shows a definite trace of dye but not enough for a colorimetric reading. Three dyes not tabulated have been tested (no. 222, Hoechst no. 229 and no. 258). All these dyes give colors in the blood serum which are too pale to be accurately estimated.

When large amounts of dye are injected into the blood stream (10 to 20 times the routine amount) the reaction is slightly different but the dyes of group I give constant figures (table 14). The vital red dyes are removed from the blood with somewhat more speed than the blue

dyes (T-1824 and T-1835, alkaline). Traces of the dye remain in the serum for days after the reading cannot be accurately determined; for example, after the injection of brilliant vital red II (table 14) the serum showed a distinct pink color on the 17th day after injection. Also a trace of this dye was excreted in the urine but the amount was too small for colorimetric reading. Following the large dose of brilliant vital red (new) the skin, mucous membranes and eye-lids became pink,

TABLE 14

Decrease in color concentration with large dosage of certain dyes. All readings in per cent

TIME AFTER INJECTION	DOG				
	19-28 (20.13 pounds)	19-136 (14.5 pounds)	19-63 (52.5 pounds)	19-117 (42.5 pounds)	19-70 (53 pounds)
	Brilliant vital red II 20 mgm. per kilo	Brilliant vital red (new) 30 mgm. per kilo	T-1824 20 mgm. per kilo	T-1835 (alkaline) 20 mgm. per kilo	Trypan blue 40 mgm. per kilo
4 min.	71	70	72	71	
40 min.	63	61	67	67	
60 min.	61	58	65	62	
2 hrs.	55	50	60	55	49
4 hrs.	41	38	50	53	33
6 hrs.	38		45	49	24
8 hrs.	36	26.6	40	44	20
12 hrs.	30	19.3	29		15
2nd day	17.3	10.0	22	30.3	10
3rd day	11.0	4.4	12	15.0	7.6
4th day	5.3	2.1	10.2	12.0	5.6
5th day	2.0	1.4	8.2	9.0	4.6
6th day	1.2		5.0	6.3	3.2
7th day			4.0		
8th day			2.8		
9th day			1.6	3.8	
10th day					1.2

which color lasted only a few minutes. Months after injections of these vital red dyes we find traces of the dye in the mesenteric and other lymph glands which are stained a delicate pink.

The curve of dye elimination is incomplete for trypan blue because the large amount used (40 mgm. per kilo) was responsible for considerable hemolysis which obscured the readings for the first two hours. It is interesting to note the long period (10 days +) during which the dye is present in the blood serum.

Several dyes of group II were tested in the same way by the use of ten times the routine dose. In these experiments the rapid elimination of the dye from the blood is very striking.

Phenolsulphonephthalein (20 mgm. per kilo) shows a 4-minute blood serum reading 19 per cent and a fall to 4 per cent at the end of 40 minutes. Only 54 per cent of the dye was recovered in the urine during 24 hours and most of this during the first hour (dog 18–60). This same animal with a routine injection gave 72 per cent elimination in the urine.

Phenoltetrachlorphthalein (20 mgm. per kilo) shows a 4-minute blood serum reading of 7 per cent and a fall to 1.4 per cent at the end of 40 minutes. With smaller amounts of dye there is scarcely a trace of dye in the serum at the end of 4 minutes. With the large dose there was noted a mere trace of dye in the urine.

Buffalo fast crimson (20 mgm. per kilo) gives a higher color per cent for the 4-minute blood serum sample (35 per cent) but complete removal at the end of 40 minutes. After the large dose the urine contained 27 per cent of the dye but after 2 mgm. per kilo dye injection we note a dye elimination in the urine of 51 to 53 per cent.

Crystal S. scarlet (20 mgm. per kilo) gives a higher blood serum 4-minute reading (39 per cent) but also complete removal in 40 minutes. The urine contained only 7 per cent of the dye which was excreted almost completely during the first hour.

DISCUSSION

A summary of all dyes of group I appears in table 9. It is readily seen that there is a remarkable average for this group of standardized dogs tested by various groups of dyes. All groups give the same average figure of blood volume per kilo body weight as 92 to 93 cc. The speed of disappearance of dye from the blood stream is constant in the average for 40 minutes and reads 11 to 13 color per cent. This is really a striking uniformity in results and for this reason these figures cannot be put aside by any quibble about the action of colloids in the blood stream, adsorption phenomena, etc.

The outstanding fact remains as follows: Any one of a large series of dyes may be injected into the blood stream and within 2 minutes it is diluted to a certain color which remains almost unchanged for 4 to 6 minutes or longer. This indicates a circulating fluid volume of a certain bulk whatever the limiting structures of this area may be. We

cannot admit that a certain portion of the dye may escape from the blood capillaries or be phagocytocized during the first 2 minutes following the injection unless we admit the same or similar possibility for the second 2-minute interval. There is no such disappearance during this second period of 2 minutes, so no person without definite proof has the right to assume or postulate any such reaction during the first 2 minutes. If these facts established by these dye injections do not harmonize with our preconceived notions concerning blood volumes and fluids circulating in the living body, then it is time to modify these concepts.

SUMMARY

Blood volume measurement is not the property of a single dye substance or group of such substances but may be accomplished by a great number of dyes.

It is essential that the dye is non-toxic, and not stored in the tissues, that the colors are such as to permit accurate determination and that the dye is removed quite slowly from the blood stream. Given these characteristics a dye is suitable for blood volume work.

It is of some significance that all suitable dyes give a remarkably constant figure on the average for blood volume determinations (table 9). This may indicate that the fluid measured in the blood stream is relatively constant, whatever our mental reservations may be concerning the actual extent of this fluid medium or its limiting structures.

A blue azo dye (T-1824) in our hands is slightly superior to the vital red groups especially as regards ease and accuracy of colorimetric readings.

Several other dyes are studied and found to be satisfactory for routine blood volume work (table 5).

A great number of dyes are included in this study and are not suitable for blood volume work. Some are rapidly removed from the blood by way of the kidney or liver. The significance of these dye reactions cannot be discussed at this time.

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BLOOD VOLUME STUDIES

IV. Blood Volume as Determined by the Change in Refractivity of the Serum Non-Protein Fraction after Injection of Certain Colloids into the Circulation

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This investigation forms a part of the general program of this laboratory which includes a study of the complex problem of blood volume determinations. This problem is being approached from all angles and other publications dealing with the subject will appear in the near future. It occurred to us that certain colloids having a high refractive index might be introduced into the blood stream with a corresponding change in the refractive index of the serum. Acacia and gelatin are used and are found to appear quantitatively in the non-protein fraction of the serum. The change in refractive index of the non-protein fraction is sufficient to permit accurate determination of the degree of dilution affected by the mixture of the colloid solution with the circulating blood. This permits us to estimate with considerable accuracy the circulating blood volume by which these colloids are diluted and in which they circulate through the body. It is significant that blood volume as determined by this method compares very closely to the blood volume as determined by the vital red dyes. This indicates that the fluid areas included in the vascular system as measured by these various substances are of similar extent.

It is not our intention to review the many papers dealing with the different phases of blood volume work. We shall refer to the work of Keith, Rowntree and Geraghty (1), who first used the "vital red" method. We shall also give frequent references and figures which cover some of the work done in this laboratory by the use of "brilliant vital red."

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The recent work of Meek and Gasser (2) is of particular interest to us. These workers introduce intravenously large amounts of acacia, then remove some blood and make the proper analysis for the contained acacia. It is necessary to hydrolyze the acacia to liberate the pentosan which can then be determined quantitatively as a phloroglucid. One of us (Davis) carried out a number of experiments using this method but in our hands this method presented certain difficulties—it is very time-consuming and the figures are not at once available. The final gravimetric determinations are carried out on such small amounts of material that slight discrepancies in weighing cause large percentage errors. We feel that the method is unique and in theory valid.

The value of the refractometer in biological analysis has been especially emphasized by Reiss (3). This work has been somewhat elaborated in this country by Robertson (4). Although various workers have solved and suggested the attack of many problems in blood analysis, we have seen no reference to the application of refractometry to the determination of colloidal additions to the blood stream in blood volume work.

A substance to be suitable for injection in our work must possess certain qualities: *a*, It should go into solution in rather high concentration. *b*, It must be relatively non-toxic and non-antigenic. *c*. It must remain unchanged in the circulation at least until thoroughly mixed. *d*, It should have a high refractive index. *e*, It should be recoverable quantitatively in the non-protein fraction of the serum.

Among the substances considered, gelatin and gum acacia were finally chosen as best suited to our work. Proteins other than gelatin were considered, but were rejected for various reasons which are obvious. Starch and inulin disappear from the blood stream too quickly. Agar agar gels too readily, is hard to get into a concentrated solution and may produce grave intoxication as demonstrated on guinea pigs by Novy and De Kruif (5). Gum tragacanth will not form a concentrated solution, and is quite expensive. Possibly other substances colloidal in nature may be suggested by other investigators if this general method finds favor.

Gelatin and acacia are both relatively cheap and easily obtained; they may be made into 20 per cent to 25 per cent solutions with little difficulty; they are non-toxic and non-antigenic, as shown by Starin (6) and others for gelatin, and as shown by Gasser and Meek (2) and by De Kruif (7) for acacia; both remain in the serum non-protein fraction; both disappear from the blood stream quite slowly as Buglia (8) has shown for gelatin, and Meek and Gasser (2) for acacia.

The fluidity of concentrated solutions of acacia is of considerable advantage; equal concentrations of gelatin readily solidify upon cooling. On the other hand, gelatin has a much higher refractive index, thus permitting somewhat smaller injections than are used with acacia. Occasionally with acacia, never with gelatin, a tendency to vomit is noted in dogs, either during or shortly after injection. This nausea is of short duration and there are no observable bad after-effects. Usually such a reaction may be avoided by very slow injection (e.g., 5 cc. per minute).

TABLE 1

Refractive indices of gelatin and acacia solutions

REFRACTIVE INDICES OF 1 PER CENT ACACIA	REFRACTIVE INDICES OE 1 PER CENT GELATIN	MIXTURE EQUAL PARTS OF 1 PER CENT ACACIA AND 1 PER CENT GELATIN
0.00132	0.00168	0.00148
0.00124	0.00160	0.00144
0.00132	0 00172	0.00152
0.00136	0.00172	
Average 0.00131	0.00168	0.00148
REFRACTIVE INDICES OF 5 PER CENT ACACIA	REFRACTIVE INDICES OF 5 PER CENT GELATIN	MIXTURE EQUAL PARTS OF 5 PER CENT ACACIA AND 5 PER CENT GELATIN
0.00655	0.00837	0.00738
0.00663	0.00833	0.00742
0.00655	0.00837	0.00742
0.00662	0.00845	
Average 0.0065875	0.00838	0.0074066+
0.0065875/5=0.0013175	0.00838/5=0.001676	0.0074066+/5=0.0014813+

Since it is sometimes advantageous to know the refractive indices of the stock solutions with which we work, we have spent some time in determining the values for diluted specimens from such solutions, and of less concentrated solutions made from the dry material. We have used commercial gum acacia, obtained in small lumps or "tears," containing an appreciable amount of foreign particles; also "Gold Label" sheet gelatin. It may very well be that chemically purified materials give somewhat different refractive indices from those which we have recorded. It will be observed that the refractive indices of 1 per cent solutions of acacia are about 0.00131 or 0.00132 greater than the solvent-distilled water, and that of gelatin is about 0.00167 or

0.00168, whether the readings are made on 1 per cent solutions or on 5 per cent readings and divided by 5. We also have determinations on 2 per cent and 10 per cent solutions which are comparable. It may be said that the readings as noted in table 1 are on separate solutions in each case, not different readings on the same solution. Our percentage solutions are made by putting the dry (desiccated) substance in a volumetric flask and making up to volume. In case of concentrated solutions (above 10 per cent at least) suitable dilutions are made to facilitate reading. It will be noted that the mixtures of equal parts 1 per cent acacia and 1 per cent gelatin give a value (0.00148 +) very close to the theoretical mean (0.001495 +) as found in the other determinations cited.

METHOD

The Pulfrich refractometer which reads the angle of total reflection to within one minute has been used for all this work. A sodium flame is used as the source of light. By means of a table the refractive indices corresponding to angles of total reflection are determined directly. Ordinarily distilled water is used as a basis for comparison. For instance, the refractive index of a 1 per cent aqueous solution of sodium chloride is 0.00160 greater than that of distilled water. The angles of total reflection of both water and salt solution as read will depend upon the temperature, the refractive index of the refractometer prism, etc., but under the same conditions of reading this difference of 0.00160 remains constant. In principle our method depends upon the difference in reading between the non-protein fraction of the blood serum before and again after the injection of a solution of a suitable colloid into the circulation.

It will be observed that in many of our experiments we have used a concentrated solution of acacia plus gelatin; that is, a solution of 20 per cent to 25 per cent acacia and 10 to 20 per cent gelatin in Locke's solution or physiological salt solution. Such a mixture is filtered through cotton, or centrifugalized to get rid of foreign material. Upon standing or centrifugalization two rather definite layers appear. The upper layer forms a soft gel upon cooling, while the lower layer never gels, though viscid, and is easily pipetted from underneath the firmer top portion. This fluid mixture we employ for injections. It remains homogeneous and has a high refractive index, the 1 to 20 dilutions reading well over 0.00200. The top layer may be warmed and injected if so desired, though we have been accustomed to use it as part of the next stock batch of gelatin-acacia mixture made up.

We usually inject (in dogs) about 1 cc. of concentrated solution per pound body weight. This amount is usually measured directly into one or more syringes. Accurate measurement of this thick, viscid fluid presents certain difficulties. The solutions are so viscous that they do not rapidly drain from a pipette. We rinse the pipette with a little warm saline solution, or draw the fluid a certain distance beyond the mark and drain only a short while. If the latter method is employed, one should calibrate the pipette for delivery at a given temperature, and should allow a constant period for drainage. Again it may be advisable to calibrate a large syringe and draw the solution up directly. If desired the solution may be warmed after measurement in the cold by laying the syringe over a steaming dish or radiator with a rubber band over the opening to prevent loss of fluid when expansion takes place.

At least three tubes should be at hand for blood samples; two of these should be clean centrifuge tubes, or test tubes, for the serum samples; and one an hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate solution for the plasma sample. A second hematocrit tube may be prepared for a plasma sample after injection, if so desired.

In dogs the blood is ordinarily withdrawn from the external jugular vein. We take from 5 to 10 cc. into a dry tube for a serum sample, and 7 to 10 cc. into oxalate for a plasma sample before injecting. The injection shou'd be made slowly, about 5 to 10 cc. per minute, the syringe rinsed with warm saline, and the washings injected. If the dog has veins easy to enter it is just as well to withdraw the needle after the injection is completed, and use the opposite side for the second blood sample. In case the veins are small and difficult to find, the needle may be kept open by slow injection of salt solution, and the second serum sample obtained on the same side. We allow 3 to 5 minutes after the injection for mixing before taking the second sample for serum.

After standing a few minutes, the clotted samples are detached from the sides of the tubes and centrifugalized for about 10 minutes for separation of the clear serum. We allow about 30 minutes at 2500 revolutions per minute for the plasma hematocrit samples.

In determining the non-protein fraction of the serum we follow the method as outlined by Robertson (4) in which the serum proteins are precipitated by N/25 acetic acid. Glass tubes 20 to 25 cm. long, having an inside diameter of at least 5 mm. and walls about 1 mm. thick, are sealed at one end. It is well to blow gently into the tube while the

sealed end is still soft, thus rounding the bottom and lessening the tendency to crack on cooling. These inexpensive tubes may be used over again after cleaning, until they become too short. Into these tubes, which have been carefully cleaned and dried, are introduced definite amounts of serum by means of any small bored pipette with a capillary tip. If possible, duplicates of each serum sample should be made. We use approximately 1 cc. amounts, although as little as 0.4 or 0.5 cc. is sufficient so long as exactly the same volume of N/25 acetic acid is added. In delivering the serum one should avoid wetting the upper part of the tube, and the formation of air bubbles.

The serum having been delivered, the same amount of N/25 acetic acid as of serum is introduced into each tube. The same pipette as used for measuring the serum, after being rinsed with distilled water and N/25 acetic acid, or a second pipette calibrated against it is used to measure the acid. In case acacia solution has been the injection fluid, the tubes of serum after injection will show much more cloudiness than the first sample, but this entirely disappears on subsequent shaking. The N/25 acetic acid solution may be made up with sufficient accuracy by diluting 4 cc. glacial acetic acid to 1750 cc.

A small glass bead is next dropped into each tube and the open end is sealed off in a flame, care being taken not to heat the contents. After cooling, the tubes are reversed a sufficient number of times to insure a thorough admixture of the contents. The tubes are next placed in a beaker containing cold water of such a depth as to completely immerse the tops of the fluid columns. It is well to pad the bottom of the beaker with cotton to guard against cracks in bumping when the water boils. The water is slowly heated to boiling and allowed to boil for 2 minutes. The tubes are then removed from the boiling water and cooled to room temperature, either in water or more slowly in air.

When the tubes have cooled, the sealed tips are broken off. The coagulum is broken up by means of a small clean wire, after which the tubes are centrifugalized for a few minutes. The clear supernatant fluid representing the non-protein fraction of the blood serum can be easily decanted or pipetted off. The samples taken before and those taken after injection are next read against each other in the refractometer. It is well to have a constant temperature in the room where the readings are done. To obtain this it may be necessary to leave the flame which is used as the source of light burning in the room for an hour or more before reading. Otherwise, at least in a long series of determinations, it may be necessary to re-read a given fluid such as

water or salt solution at frequent intervals to find the change due to rising temperature. Between separate samples the cup of the refractometer should be rinsed with distilled water and carefully dried with absorbent cotton or filter paper, preferably followed by lens paper.

We consider that the actual reading of the refractometer is the simplest step in the whole method. With a little practice anyone can bisect the opposite angles of the X with the well demarcated line between light and shadow shifted by the fine adjustment screen. It is better for one individual to make all the readings in a given experiment, since possible slight errors are then more likely to offset each other. The angles as read on the scale are referred to a table giving the corresponding refractive indices, and the difference between solutions is obtained at once by subtraction.

The plasma percentage before injection is calculated from the hematocrit tube readings, allowance being made for the oxalate solution previously in the tube. The total plasma withdrawn before injection is subtracted from the total fluid volume injected; the result we assume to be the increment to the plasma volume caused by injection.

A suitable dilution (e.g., 1 to 20) is made of the concentrated injection fluid and its increased refractivity over the salt solution solvent is determined.

We now calculate:

$$\frac{\text{Amount Injected} \times \text{Reading of Diluted Sample} \times \text{Dilution}}{\text{Difference in Refractivity of Non-Proteins}} = \text{Increased Plasma Volume}$$

Plasma Volume Before Injection = Increased Plasma Volume − Plasma Increment.

$$\text{Blood Volume} = \frac{\text{Plasma Volume (Before Injection)}}{\text{Plasma Per Cent}} \times 100.$$

The difference in refractivity of non-proteins is obviously twice the difference in refractometer readings, since each serum sample is diluted one-half with N/25 acetic acid.

In case simple solutions of gelatin or acacia are used the plasma volume may be determined as follows: Knowing the reading of a true 1 per cent solution, and that of an accurately diluted sample of the concentrated injection fluid, the number of grams injected may be determined. From the difference in the non-protein reading before and after the injection the concentration per cubic centimeter of plasma

can be calculated. Then the grams injected divided by the plasma concentration minus the plasma increment should give the same value as arrived at in the above formula. In case an hematocrit is taken after injection the above figuring may be done without subtracting the plasma increment and the larger result divided by the plasma per cent after injection; the result is probably somewhere near the blood volume after injection. From this the total volume injected minus the total blood withdrawn may be subtracted, and the resulting figure is often very close to the blood volume before injection as determined by the first formula. However, we consider the first method to be the more reliable.

In these calculations we are considering the vascular tree as a definite container with impermeable walls, into which a known fluid is injected, the whole mixed, and the total contents determined by the concentration of injected material in a given sample of the diluted fluid. Obviously we are not justified in these assumptions; indeed it is occasionally found that the plasma percentage in the sample after injection is less than it was beforehand. No doubt there are changes occurring in the blood stream that are quite beyond our control and about many of which we know nothing. There have been observations in this laboratory which indicate a frequent leucopemia after injection of acacia; sometimes there seems to be a definite delay of clotting after injection of acacia although *in vitro* it requires practically an equal volume of 10 per cent acacia to prevent clotting. However, other blood volume methods meet the same or similar difficulties, in that changes going on *in vivo* are not fully understood.

EXPERIMENTAL OBSERVATIONS

It must be demonstrated at once that acacia and gelatin alone or combined are not carried out of the blood plasma by blood coagulation. It must be shown that these two colloids may be recovered quantitatively from the non-protein fraction of the blood serum. Our experiments have convinced us that acacia and gelatin are not disturbed by blood coagulation and do appear quantitatively in the non-protein fraction of the blood when this method is employed as described. Experiment 1 may be taken as a typical example.

Experiment I. Recovery of acacia and gelatin in vitro. The following solutions were prepared:

1. One cubic centimeter 10 per cent acacia in NaCl solution + 9 cc. whole blood.

2. One cubic centimeter NaCl solution + 9 cc. whole blood. (Control.)
3. One cubic centimeter approximately 10 per cent gelatin in NaCl solution + 9 cc. whole blood.
4. One cubic centimeter NaCl solution + 1 cc. gelatin and acacia mixture in NaCl solution + 8 cc. whole blood.
5. Two cubic centimeters NaCl solution + 8 cc. whole blood. (Control.)
6. One cubic centimeter 10 per cent acacia in NaCl solution + 9 cc. NaCl solution.
7. One cubic centimeter approximately 10 per cent gelatin in NaCl solution + 9 cc. NaCl solution.
8. One cubic centimeter gelatin and acacia mixture in NaCl solution + 19 cc. NaCl solution.
9. NaCl solution.

Non-protein readings:

	Angle of Ref.	*Index*
1.	66°6′ 66°6′	1.33855
2.	66°26′ 66°26′	1.33695
3.	66°4′ 66°4′	1.33871
4.	65°50′ 65°50′	1.33983
5.	66°26′ 66°26′	1.33695

Other solutions:

6.	66°4′30″ 66°4′30″	1.33867
7.	66°3′ 66°3′	1.33879
8.	66°6′ 66°6′	1.33855
9.	66°21′ 66°21′	1.33735

Blood plasma percentage = 41.65 per cent.

1.33855 (1) − 1.33695 (2) = 0.00160; 0.00160 × 2 = 0.00320.

0.00320 × 0.4165 = 0.0013328, reading of 1 per cent acacia in the whole blood.

1.33867 (6) − 1.33735 (9) = 0.00132, reading of 1 per cent acacia in NaCl solution.

(100 × 0.0013328 − 0.00132)/0.00132 = 0.9 per cent difference.

1.33871 (3) − 1.33695 (2) = 0.00176; 0.00176 × 2 = 0.00352.
0.00352 × 0.4165 = 0.00146608, 1 per cent gelatin in whole blood.
1.33879 (7) − 1.33735 (9) = 0.00144, 1 per cent gelatin in NaCl solution.
(100 × 0.00146608 − 0.00144)/0.00144 = 1.7 per cent difference.

1.33983 (4) − 1.33695 (5) = 0.00288; 0.00288 × 2 = 0.00576.
0.00576 × 0.4165 = 0.00239904, reading of 1:10 dilution of gelatin and acacia mixture in blood.
1.33855 (8) − 1.33735 (9) = 0.00120, reading of 1:20 dilution of mixture.
2 × 0.00120 = 0.00240, reading of 1:10 dilution of gelatin and acacia mixture in NaCl solution.
(100 × 0.00240 − 0.00239904)/0.00240 = 0.04 per cent difference.

It will be seen that within experimental error the recovery of acacia and gelatin was complete in whole blood. Where the gelatin and acacia were used separately a slight positive error occurred, and when a mixture of the two was employed the check was almost perfect.

For a considerable range of angles the index per minute is 8 in the 5th decimal place; hence the difference between two solutions is the difference in minutes × 0.00008. As:

$$66^\circ 26' - 66^\circ 6' = 20'; \; 20 \times 0.00008 = 0.00160$$

In ordinary calculations we seldom use decimals in referring to refractive indices, thus for convenience we generally call that of acacia simply 132, and gelatin 168, etc.

It has been of interest to determine whether the method can be used with accuracy in the presence of hemolysis, jaundice, lipemia, etc. These conditions sometimes offer considerable difficulties in dye determinations of blood volume. We have found that these factors introduce no error in the refractometric method. Hemoglobin is entirely precipitated with the proteins. We have never taken any special precautions to prevent hemolysis, consequently a moderate grade has sometimes occurred. Such experiments have always been tabulated as normal, without question. Bile pigments and fat even in excessive amounts are inconsequential factors after the processes of dilution and precipitation.

The following experiment illustrates recovery in the presence of bile.

Experiment II. Recovery of media in the presence of bile. Sample: 100 cc. fresh blood + 5 cc. dog's bile.

The following solutions were prepared:

1. Nine cubic centimeters serum (containing bile) + 1 cc. 0.9 per cent NaCl solution.

2. Nine cubic centimeters serum (containing bile) + 1 cc. gelatin and acacia mixture in 0.9 per cent NaCl solution—(3 samples).

3. Nine cubic centimeters 0.9 per cent NaCl solution + 1 cc. gelatin + acacia mixture (2 samples).

4. Nine hundredths per cent NaCl solution.

Non-protein readings:

	Angle of Ref.	
1	66°38′30″ 66°38′	Average 66°38¼′
2	66°11′30″ 66°11′30″ 66°11′	Average 66°11⅓′

Difference (1 and 2) = 26 1/12′; 26 1/12 × 8 × 2 = 430⅔.

Readings of media:

	Angle of Ref.	
3	65°27′ 65°26′30″	Average 65°26¾′
4	66°20′	

Difference (3 and 4) = 53¼′; 53¼ × 8 = 426.

100 × 430⅔ − 426 / 426 = 1.09 per cent error.

It will be seen at once that the difference in recovery in NaCl solution and in jaundiced serum lies well within the experimental error of measurement and reading, and that even so, the error as noted is in favor of the serum mixture.

The following two experiments were performed upon the same dog, one during an induced lipemia, the other in a normal period. We have another experiment on the same animal on still another date which gives practically the same blood volume which these experiments show.

Experiment III. Blood volume in lipemia. August 13, 1919.

Dog 20-6. Young male adult, in good condition. Weight 23¼ pounds (10.52 kgm.). Gave 50 cc. cottonseed oil + 100 cc. milk by stomach tube 1½ hours before withdrawal of blood.

Removed 16 cc. blood before injection. Plasma per cent = 57.

Injected 24½ cc. media + 20 cc. NaCl solution. Removed 18 cc. after 6 to 7 minutes.

Serum shows marked lipemia.

Media diluted 1:20 − reading 66°2′	18′ difference
NaCl solution − reading 66°20′	18 × 8 = 144
Serum non-protein, before injection − reading	66°19¾′ 66°19¼′

Serum non-protein after injection — reading..... $\begin{cases} 66°13\frac{1}{2}' \\ 66°13\frac{1}{2}' \end{cases}$

Difference = $6\frac{1}{4}'$; $6\frac{1}{4} \times 8 \times 2 = 100$.

44.5 cc. total injection
9.0 cc. plasma withdrawn
35.5 cc. plasma increment

$$\frac{144 \times 24\frac{1}{2} \times 20}{100} - 35.5 = 670 \text{ cc. plasma volume.}$$

670 / 0.57 = 1176 cc. blood volume.
1176 / 10520 = 11.1 cc. per 100 grams body weight.

Experiment IV. August 27, 1919.

Dog 20–6. Young male adult (see exper. III). Weight 24 pounds (10.9 kgm.).

Removed 16 cc. blood before injection. Plasma = 54.7 per cent.

Injected 25 cc. acacia + gelatin mixture (previously found to read 164 in a 1:25 dilution) + 14 cc. NaCl solution.

Dog became a little sick but did not vomit.

Second blood sample withdrawn 4 – 5 minutes after injection.

Angle of Ref.

1. Non-protein—before injection..... $\begin{cases} 66°19'30'' \\ 66°19'30'' \\ 66°20' \end{cases}$

2. Non-protein—after injection..... $\begin{cases} 66°11' \\ 66°11' \\ 66°11'+ \\ 66°11'+ \end{cases}$

Angle of Ref.

No. 1 (before injection) re-read because of rising temperature..66°21'

Difference about $9\frac{1}{2}$ ($66°20\frac{1}{2}' - 66°11'$)

$$9\frac{1}{2} \times 8 \times 2 = 152$$

39 cc. total injection − 9 cc. plasma withdrawn = 30 cc. plasma increment.

$$\frac{164 \times 25 \times 25}{152} - 30 = 674 \text{ cc. plasma volume.}$$

674 / 0.547 = 1177 cc. blood volume.
1177 / 10900 = 10.7 cc. per 100 grams body weight.

That the blood volumes as determined in experiments III and IV so nearly coincide is a mere chance, especially when the time interval between determinations, the change in weight, etc., are considered. Nevertheless, the results certainly suggest that even a marked lipemia does not affect the non-protein readings.

With the possibility of error from these more or less abnormal conditions eliminated, we may go on to the consideration of normal cases.

Table 2 gives the results of blood volume determinations in a number of dogs. It is scarcely necessary to give protocols of the individual experiments; the essential details are given in the table, and experiments III and IV are indicative of the usual procedure and calculations.

Most of these dogs were in good condition, a few were very anemic as noted in the remarks.

In nearly every case we have dye determinations on the animal; some of these were done in the regular course of experiments by various laboratory workers, others were kindly performed by either Mr. H. P. Smith or Mrs. Robscheit, especially to serve as checks for our own experiments.

Blood volumes done by the dye method at an interval of several days or weeks from the refractometric method are of little comparative worth; the determinations done on the same day are of the greatest value. Sometimes the difference between the two results is rather large, but on the other hand, the similarity is often quite striking. We were pleased to find that the refractometric method sometimes gives higher results, sometimes lower than the dye method. This lack of constancy probably indicates the occurrence of experimental errors rather than a fundamental fault in the method. The ratio of blood volume to body weight is quite similar in the two methods. The average amount of blood per 100 grams body weight in the twenty-one experiments tabulated in table 2 is 9.76 cc.

In a series of dogs reported by Meek and Gasser (2) using the acacia-phloro-glucid method, the blood volume in per cent of body weight was 9.74 per cent. To compare with this, our results as given should be multiplied by the specific gravity of blood, but even so the similarity is rather striking. The plasma percentages of body weight in dogs as determined by Keith, Rowntree and Geraghty (1) are quite comparable to our results.

Reference to table 2 shows that we gave much larger injections in the early experiments than we did later. The later work was largely done with the solutions of high refractive indices. Furthermore, we came to the conclusion that large volumes of fairly concentrated colloids produced greater upset of equilibrium (i.e., changes in cell content and ratio, unaccountable plasma percentages, disturbances in protein content, etc.) than did small injections, and were to be avoided when possible. We attempted to obtain the blood volume in a few instances when Mr. Smith and Mr. Arnold injected very large amounts of 6 per cent acacia without previously producing an anemia. In two experi-

TABLE 2

Blood volume results—refractometric method—dogs

EXPERIMENT NUMBER	DOG NUMBER	DATE	WEIGHT	INJECTION	PLASMA BEFORE INJECTION	PLASMA VOLUME	BLOOD VOLUME	BLOOD PER 100 GRAMS BODY WEIGHT	BLOOD VOLUME DYE METHOD	REMARKS
			kgm.		*per cent*	*cc.*	*cc.*	*cc.*		
V	18–114	7/25/18	11.64	60 cc. 20 per cent acacia	49	611	1247	10.7		See next experiment
VI	18–114	8/3/18	11.50	60 cc. 20 per cent acacia	44.4	547	1232	10.7	1294	Dye determination made August 14
VII	18–113	7/25/18	12.44	60 cc. 20 per cent acacia	46.8	491	1049	8.4	940 (1) 1136 (2)	Dye determinations made (1) June 10, and (2) August 14
VIII	18–115	8/1/18	7	50 cc. 20 per cent acacia	60.58	461	760	10.8	761 (1) 1000 (2)	Dog is in poor condition. Dye determinations (1) June 10, and (2) September 19
IX	18–24	3/12/19	13.6	54½ cc. 20 per cent acacia		582	1294	9.5	1305	Calculated volume after injection. Dye determination also after injection
X	18–39	3/14/19	11.1	45 cc. 20 per cent acacia		553	1270	11.4	1255 (1) 1205 (2)	Calculated volume after injection Dye determinations (1) before and (2) after injection
XI	18–4	3/18/19	20.5	86 cc. 20 per cent acacia	37.1	804	2167	10.5	2264 (1) 2117 (2)	Dye determinations (1) before and (2) after injection of acacia

XII	19–11	4/15/19	20	64 cc. 25 per cent acacia	47	854	1817	9.1	1955 (1) 2630 (2)	Dye determinations (1) before and (2) after injection of acacia
XIII	17–157	8/8/19	10.02	34½ cc. 20 per cent acacia	50	514	1028	10.2	1123	Dye determination made on a different day, same week
XIV	18–38	7/?/19	10.68	34 cc. mixture gelatin and acacia	48.21	464	962	9.0	903	Dye injected in acacia solution
XV	18–38	8/4/19	10.45	25 cc. mixture gelatin + acacia	50.8	513	1009	9.5		See previous experiment
XVI	16–160	8/9/19	12.045	24½ cc. mixture gelatin + acacia	45.2	540	1323	10.9	1040	Dye determination made August 18
XVII	18–126	8/12/19	10.795	24½ cc. approximately 16 per cent gelatin	46.9	556	1185	10.9	975	Dye determination made August 18
XVIII	19–93	8/22/19	12.727	25 cc. mixture gelatin + acacia	68.5	795	1160	9.1	1023	Dog anemic. Dye determination made on previous day
XIX	19–94	8/22/19	13.80	25 cc. mixture gelatin + acacia	70	909	1298	9.4	1090	Dog anemic. Dye determination made on previous day
XX	19–95	8/23/19	13.98	25½ cc. mixture gelatin + acacia	63	718	1139	8.1	1106	Dog anemic. Dye determination made two days previously
XXI	19–96	8/23/19	14.204	25½ cc. mixture gelatin + acacia	61.2	642	1049	7.3	993	Dog anemic. Dye determination made two days previously

TABLE 2—*Concluded*

EXPERIMENT NUMBER	DOG NUMBER	DATE	WEIGHT	INJECTION	PLASMA BEFORE INJECTION	PLASMA VOLUME	BLOOD VOLUME	BLOOD PER 100 GRAMS BODY WEIGHT	BLOOD VOLUME DYE METHOD	REMARKS
			kgm.		*per cent*	*cc.*	*cc.*	*cc.*		
XXII	19–84	8/25/19	9.432	20 cc. mixture gelatin + acacia	41.7	434	1040	11.0	996	Dye injected with acacia-gelatin mixture
XXIII	19–83	8/25/19	14.9	25 cc. mixture gelatin + acacia	44.82	665	1484	9.96	1545	Dye injected with acacia-gelatin mixture
XXIV	16–158	8/25/19	12.1	25 cc. mixture gelatin + acacia	72.5	639	882	7.3	929	Dye injected with acacia-gelatin mixture
XXV	18–114	8/27/19	13.8	25 cc. mixture gelatin + acacia	53.1	829	1561	11.3	1480	Dye injected with acacia-gelatin mixture
Average								9.76		

ments out of about half a dozen we obtained results comparable with the dye method. Such enormous injections are certainly unnecessary and unfavorable for subsequent refractometric determinations. Experiment XXVI shows one of the few good results which we obtained.

Experiment XXVI. Blood volume determined following the injection of a large amount of 6 per cent acacia solution. August 8, 1919.

Dog 17–38. Weight 28 pounds (12.72 kgm.).

Mr. Arnold injected 635 cc. of approximately 6 per cent acacia; this was the estimated plasma volume.

Sample following injection was removed in 5 minutes.

A 1:6 dilution of the injection media read 120.

Plasma per cent = 42.5 immediately preceding injection.

Difference in non-protein readings before and after = $22\frac{1}{2}$; $22\frac{1}{2} \times 8 \times 2 = 360$.

$$\frac{120 \times 6 \times 635}{360} - 635 = 635 \text{ plasma volume before injection.}$$

635 / 0.425 = 1496 cc. blood volume.

Mr. Arnold obtained the following results by the dye method:

	Plasma volume	*Blood volume*
Before injection	585	1416
After injection	901	1567

In addition to experiments on dogs we have a few observations on rabbits.

With these animals we use an improvised box holder with side holes through which the ears are drawn. Blood is obtained from the marginal ear vein through a small longitudinal slit made with a safety razor blade. Collection is made into small tubes, one for plasma and one for serum, as with dogs. Three cubic centimeter hematocit tubes are made from sections of straight-sided, graduated 10 cc. pipettes; one end of a section is sealed off, and the graduations subsequently verified or modified if necessary. To prevent clotting 0.4 or 0.5 cc. of 1.6 per cent oxalate solution is used per tube. To dilate vessels and hasten bleeding an electric light bulb held almost touching the ear is found very satisfactory. Injection of the colloidal material is made into any available ear vein from a syringe with a small needle, and collection of blood sample after injection made from the marginal vein of the opposite ear. Using this technique little blood need be taken, and many determinations may be made upon the same animal with minimal injury. Table 3 gives the results on four rabbits.

The amounts of solution injected have always been quite small and no untoward reactions have been noted. The checks with dye determinations differ by 4.8 per cent to 11 per cent.

TABLE 3

Blood volume results on rabbits—refractometric method

EXPERIMENT NUMBER	RABBIT NUMBER	DATE	WEIGHT	INJECTION	PLASMA BEFORE INJECTION	PLASMA VOLUME	BLOOD VOLUME	BLOOD PER 100 GRAMS BODY WEIGHT	BLOOD VOLUME DYE METHOD	REMARKS
			grams		*per cent*	*cc.*	*cc.*	*grams*		
XXVII	I	7/23/19	3375	10 cc. 20 per cent acacia	60	149	248	7.3	260	Samples removed during period 8 to 17 minutes after injection. Dye determination made on same day
XXVIII	I	7/29/19	3375	9.5 cc. 20 per cent gelatin	64.8	159	245	7.2		Sample removed 10 minutes after injection
XXIX	I	8/7/19	3300	5 cc. concentrated mixture gelatin + acacia + 5 cc. 0.9 per cent NaCl solution	60.5	125	206	6.2		Sample removed 5 minutes after injection
XXX	I	8/24/19	3385	5 cc. concentrated mixture gelatin + acacia + 5 cc. 0.9 per cent NaCl solution	62.7	145	231	6.8		Sample removed 5 minutes after injection
XXXI	II	7/24/19	3100	10 cc. mixture gelatin + acacia	65.66	154	250	8.06	220 (1) 226 (2	Sample removed 30 minutes after injection. Dye determinations made on same day (1) with ear veins (2) femoral vein and artery

XXXII	II	8/14/19	3150	10 cc. mixture gelatin + acacia	54.7	110 (1) 122 (2)	201 (1) 223 (2)	6.3		(1) Sample removed 5 minutes after injection (2) Sample removed 30 minutes after injection
XXXIII	III	7/21/19	3250	10 cc. mixture gelatin + acacia	62.8	144	229	7.0	244	Sample removed about 15 minutes after injection
XXXIV	III	8/5/19	3190	10 cc. mixture gelatin + acacia	58.3	112	193	6.05		Sample removed 5 minutes after injection
XXXV	III	8/15/19	3175	10 cc. mixture gelatin + acacia	59	118	200	6.3		Sample removed 5 minutes after injection
XXXVI	IV	7/22/19	2750	8 cc. mixture gelatin + acacia	57.7	130	225	8.1	200	Dye injected with gelatin-acacia mixture. Sample removed about 15 minutes after injection
XXXVI	IV	7/30/19	2650	4 cc. concentrated gelatin + acacia mixture + 6 cc. 0.9 per cent NaCl solution	62.6	136	217	8.1		Sample removed 30 minutes after injection
XXXVIII	IV	8/9/19	2600	10 cc. mixture gelatin + acacia	60.3	113	188	7.2		Sample removed 5 minutes after injection
XXXIX	IV	8/18/19	2650	10 cc. mixture gelatin + acacia	57	119	209	7.8		Sample removed 15 minutes after injection
XL	IV	8/30/19	2550	7.5 cc. 20 per cent acacia	58.1	97	167	6.6		Sample removed 5 minutes after injection
Average								7.07		

The large ratio of blood volume to weight is rather interesting. We think that without doubt the blood volume as determined by samples obtained 5 minutes after injection should be considered the most accurate. Averaging the values obtained from these 5-minute samples we find that the average of 7.07 cc. of blood per 100 grams body weight is reduced to 6.49. Even this is much higher than the values usually accepted. Meek and Gasser (2) give an average of 5.44 per cent of the body weight, and other observers even less.

We have several experiments on rabbits where blood samples were withdrawn at varying intervals, which show increasing blood volumes as time goes on. This, of course, is due to at least two factors: *a*, the actual elimination of acacia, and *b*, the dilution of the plasma, presumably by fluid drawn into the blood stream from the tissues because of increased osmotic pressure. The following abbreviated protocol of experiment XXXV shows the changes mentioned.

Experiment XXXV. Blood volume. August 15, 1919.

Rabbit III. Weight 3175 grams.

Injected 10 cc. gelatin + acacia mixture (reading 1: 12½ dilution = 0.00172) and 3 cc. of 0.9 per cent solution NaCl.

I. Sample before injection. Plasma per cent = 59. (Removed 4.35 cc. blood). Non-protein readings $\begin{cases} 66°25' \\ 66°25' \end{cases}$

II. Sample removed 5 minutes after injection. Non-protein readings $\begin{cases} 66°15\frac{1}{2}' \\ 66°15\frac{1}{2}' \end{cases}$

III. Sample removed 15 minutes after injection. Non-protein readings $\begin{cases} 66°16' \\ 66°16' \end{cases}$

IV. Sample removed 30 minutes after injection. Plasma per cent = 61. Non-protein readings $\begin{cases} 66°17\frac{1}{2}' \\ 66°17\frac{1}{2}' \end{cases}$

V. Sample removed 1 hour after injection. Plasma per cent = 63. Non-protein readings $\begin{cases} 66°18\frac{1}{2}' \\ 66°18\frac{1}{2}' \end{cases}$

VI. Sample removed 20 hours after injection. Plasma per cent = 61.7. Non-protein readings $\begin{cases} 66°22' \\ 66°22' \end{cases}$

Altogether, 23.27 cc. blood were removed in 10 samples.

Calculating as in previous experiments the results are approximately:

SAMPLE	PLASMA VOLUME	BLOOD VOLUME	PER 100 GRAMS BODY WEIGHT
5 minutes	118	200	6.3
15 minutes	124	210	6.6
30 minutes	148	241	7.5
1 hour	169	268	8.4

It will be noted that the plasma percentage increased for at least an hour. Such variations in values with lapse of time inclines us to accept the results obtained from 5-minute samples. This probably gives enough time for mixing and a minimal time for elimination and dilution. The reading of the sample after 20 hours indicates that an appreciable amount of injection media is still in the circulation. We have numerous observations of a similar nature showing that total elimination is slow, and corroborating the results of previous workers.

We have done very little work with cats because of a scarcity of animals. Our technique has been to inject into the femoral vein, and withdraw blood from the femoral artery through a cannula. Our experiments are so few in number that we consider it unwise to report these experiments at this time.

DISCUSSION

The colloid-refractometric blood volume method as outlined gives results quite comparable to the dye methods, and to Meek and Gasser's acacia method. This method requires a slightly longer time to complete than the dye method but it is much shorter than the method of Meek and Gasser. A routine determination can be finished in about two hours.

We have considered reading the plasma or serum difference without precipitating the proteins, but the dilution of the protein content makes such determination altogether unreliable. An alternate possibility, however, is to use the plasma non-proteins, which necessitates only one blood sample before injection and one afterward. A serious drawback to such a procedure is that the samples put into oxalate solution must be measured and of exactly equal volume in order that one may be read against the other; also the dilution with oxalate must be considered in computations. However, this method can be employed in conditions in which the blood for any reason fails to clot or clots incompletely.

We have tried double blood volume determinations, that is, on the basis of two separate injections at short intervals, only twice (on rabbits) and then without success. The recovery after the second injection was less than was to be expected in these instances. We can offer no explanation of this failure; probably further work with dogs as well as rabbits will clear up the difficulty.

If Meek and Gasser (2) can recover acacia quantitatively as phloroglucid after a second injection, presumably there should be no difficulty in recognizing it refractometrically.

It has occurred to us that the transient white cloudy precipitate formed upon adding dilute acetic acid to blood serum containing acacia might be used as a qualitative method in studying the elimination curve of acacia from the blood stream.

The injection media and materials used in this method are comparatively cheap and are always available. To laboratories equipped with a refractometer we offer the procedure as worthy of trial. The time needed for a complete determination is not too long for ordinary work, the individual steps are relatively simple, and the results compare favorably with those obtained by other methods. A point of considerable advantage is the small amount of blood needed for the determinations; if necessary, accurate results should be obtained with one cubic centimeter of serum before, and one after injection, and a micro-hematocrit reading. Another point of value in the method is that hemolysis, lipemia and cholemia are not disturbing factors.

SUMMARY

We have outlined a method of determining blood volume which consists essentially in reading refractometrically the serum non-protein increase after the intravenous injection of a known amount of acacia or gelatin solution, or a mixture of the two.

By this method we have found in the dog an average of 9.76 cc. blood per 100 grams body weight. In the rabbit an average of determinations using samples removed 5 minutes after injection of media gives a volume of 6.49 cc. per 100 grams body weight.

Of great importance is the fact that hemolysis, lipemia and cholemia do not affect the accuracy of determinations by this method.

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THE INFLUENCE OF SPLENIC EXTRACT ON THE NUMBER OF CORPUSCLES IN THE CIRCULATING BLOOD

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These experiments were undertaken in order to determine the effect on the number of both red and white corpuscles in the circulating blood of injections of splenic extract. It is believed that such a determination must throw some light on the possibility of the presence of an internal secretion of the spleen capable of affecting formation or destruction of blood corpuscles.

For reasons stated elsewhere (1) rabbits were used in all cases and, to exclude so far as possible changes in the corpuscle content of the blood accompanying digestion, the animal being used was deprived of food and water during the course of the experiment. The procedure followed was to make an initial count of both red and white corpuscles and then to inject subcutaneously the desired dose of splenic preparation. The dose was dissolved in physiological saline solution and warmed to 37°C. before injection. Subsequent red and white corpuscle counts were made at half-hour intervals during the first two or three hours after injection and a final count was made four or five hours from the beginning of the experiment. The diluting fluids used in making the corpuscle counts were 0.7 per cent sodium chloride solution for the erythrocytes and 0.5 per cent acetic acid for the leucocytes. The counts were made in the usual manner with Levy haemacytometers.

In the first series of experiments the preparation employed was powdered dried spleen, prepared by Armour and Company, one gram of which is said to represent approximately five grams of fresh spleen of the calf. The dose used was 10 mgm. of this powder per kilogram of body weight of the rabbit, dissolved in about 2 cc. of physiological saline solution and injected subcutaneously. The protocol of a typical experiment of this group follows:

Experiment 2, November 3, 1918

10.42 a.m. Red blood corpuscles 7,264,000 per cubic millimeter. White blood corpuscles 9,200 per cubic millimeter.
10.45 a.m. 10 mgm. powdered dried spleen (Armour and Company) per kilogram of body weight injected subcutaneously.
11.15 a.m. Red blood corpuscles 6,800,000 per cubic millimeter. White blood corpuscles 7,000 per cubic millimeter.
11.45 a.m. Red blood corpuscles 6,440,000 per cubic millimeter. White blood corpuscles 7,000 per cubic millimeter.
12.15 p.m. Red blood corpuscles 6,704,000 per cubic millimeter. White blood corpuscles 6,200 per cubic millimeter.
12.45 p.m. Red blood corpuscles 6,944,000 per cubic millimeter. White blood corpuscles 7,200 per cubic millimeter.
2.45 p.m. Red blood corpuscles 6,976,000 per cubic millimeter. White blood corpuscles 14,100 per cubic millimeter.

TABLE 1

Decrease in erythrocyte count after injection of powdered dried spleen (Armour and Company), 10 mgm. per kilogram of body weight

EXPERIMENT NUMBER	INITIAL COUNT	MINIMAL COUNT	AMOUNT OF DECREASE	PERCENTAGE EFFECT	MINIMUM IN	DURATION OF EFFECT
					minutes	*minutes*
1	6,424,000	6,352,000	72,000	1.12	30	30
2	7,264,000	6,440,000	824,000	11.34	60	90
3	7,168,000	6,336,000	832,000	11.60	90	120
4	6,624,000	6,256,000	368,000	5.55	30	60
5	7,104,000	6,608,000	496,000	6.98	30	90
6	6,144,000	6,080,000	64,000	1.04	60	90
7	6,960,000	6,160,000	808,000	12.93	30	60
8	7,175,000	6,960,000	215,000	2.99	30	60
9	7,488,000	7,144,000	344,000	4.59	30	30
10	6,424,000	6,376,000	48,000	0.74	60	60
Averages	6,877,500	6,471,200	406,300	5.88	45	69

There are ten experiments in this group, in each of which the result was practically the same—a slight reduction in the red corpuscle count of short duration (table 1), a prompt reduction in the number of white corpuscles followed in several hours by a considerable leucocytosis. The change in the leucocyte count is identical with the effect described by Wells (2) and others as following upon the subcutaneous injection of any foreign protein and can not, therefore, be considered a specific effect of spleen unless it is still obtained after the administration of a protein-free preparation.

We attempted to obtain a protein-free splenic preparation by precipitation with alcohol. A 2.5 per cent solution in distilled water of the powdered dried spleen was made and poured in a thin stream into absolute alcohol. The proportion of solution to alcohol used was so arranged that the resulting mixture always contained at least 80 per cent of alcohol. The precipitate thus formed was filtered off and the alcoholic filtrate evaporated to dryness on a water bath. Five grams of the powdered spleen yielded 1.5 grams of final residue or about 60 mgm. per gram of fresh spleen. While realizing that this residue is very probably not rendered absolutely protein free by the method employed, we will for convenience speak of it hereafter as protein-free splenic preparation (Armour and Company). In the second series of experiments this residue was used in the dose of 10 mgm. per kilogram of body weight of the rabbit. The protocol of one of the experiments of this group follows:

Experiment 14, January 27, 1919

10.20 a.m. Red blood corpuscles 6,960,000 per cubic millimeter. White blood corpuscles 7,000 per cubic millimeter.

10.25 a.m. 10 mgm. protein-free splenic preparation (Armour and Company) per kilogram of body weight injected subcutaneously.

10.55 a.m. Red blood corpuscles 5,712,000 per cubic millimeter. White blood corpuscles 8,400 per cubic millimeter.

11.25 a.m. Red blood corpuscles 5,632,000 per cubic millimeter. White blood corpuscles 7,000 per cubic millimeter.

11.55 a.m. Red blood corpuscles 5,816,000 per cubic millimeter. White blood corpuscles 8,500 per cubic millimeter.

12.25 p.m. Red blood corpuscles 6,624,000 per cubic millimeter. White blood corpuscles 11,600 per cubic millimeter.

12.55 p.m. Red blood corpuscles 6,816,000 per cubic millimeter. White blood corpuscles 8,200 per cubic millimeter.

2.55 p.m. Red blood corpuscles 7,200,000 per cubic millimeter. White blood corpuscles 6,800 per cubic millimeter.

Again we find that the red corpuscle count decreased following the injection (table 2), the decrease in these experiments being much more marked and more persistent than in the first series. The characteristic effect of foreign protein injections on the white corpuscles is no longer in evidence. On the other hand, an increased leucocyte count of brief duration is recorded in every experiment, occurring at or about the time of maximum decrease of the red corpuscles (table 3). We feel justified, therefore, in concluding that the effect on the erythrocyte count at least is a specific effect of the splenic preparation employed.

We now proceeded to make fresh extracts of the spleen of dogs and cats. The fresh spleen was ground with sand and macerated over night with twice its bulk of physiological saline solution. The prepara-

TABLE 2

Decrease in erythrocyte count after injection of protein-free splenic preparation (Armour and Company), 10 mgm. per kilogram of body weight

EXPERIMENT NUMBER	INITIAL COUNT	MINIMAL COUNT	AMOUNT OF DECREASE	PERCENTAGE EFFECT	MINIMUM IN	DURATION OF EFFECT
					minutes	*minutes*
11	6,336,000	4,672,000	1,664,000	26.26	90	150
12	6,672,000	5,664,000	1,008,000	15.10	60	90
13	7,328,000	6,480,000	848,000	11.57	60	90
14	6,960,000	5,632,000	1,328,000	19.08	60	120
15	6,800,000	4,992,000	1,080,000	26.58	90	120
16	7,232,000	6,248,000	984,000	13.60	60	90
17	6,272,000	4,384,000	1,888,000	30.10	60	90
18	5,360,000	4,800,000	560,000	10.44	60	90
19	6,940,000	5,456,000	1,484,000	21.38	60	120
20	6,456,000	5,632,000	824,000	12.76	60	60
Averages	6,635,600	5,396,000	1,239,600	18.68	66	102

TABLE 3

Increase in leucocyte count after injection of protein-free splenic preparation (Armour and Company), 10 mgm. per kilogram of body weight

EXPERIMENT NUMBER	INITIAL COUNT	MAXIMAL COUNT	AMOUNT OF INCREASE	PERCENTAGE EFFECT	MAXIMUM IN
					minutes
11	6,400	10,500	4,100	64.06	150
12	8,800	13,600	4,800	54.54	120
13	11,000	15,200	4,200	38.18	60
14	7,000	11,600	4,600	65.71	120
15	6,200	15,500	9,300	150.00	60
16	13,300	16,600	3,300	24.06	60
17	11,100	13,000	1,900	17.11	90
18	10,900	12,800	1,900	17.43	90
19	12,000	15,000	3,000	25.00	30
20	11,500	14,800	3,300	28.69	30
Averages.....	9,820	13,860	4,040	41.14	81

tion was then strained through muslin and filtered. The filtrate was poured in a thin stream into absolute alcohol, care again being taken not to reduce the alcohol below 80 per cent. The precipitate was

filtered off and the alcoholic extract evaporated to dryness on the water bath. We obtained from dog's spleen about 26 mgm. of final residue per gram of fresh spleen and from cat's spleen about 10 mgm. per gram of fresh spleen.

Five experiments were performed in each of which 10 mgm. of the extract of dog's spleen per kilogram of body weight were injected subcutaneously, and two experiments in each of which a similar dose of the extract of cat's spleen was administered. The results so far as the red corpuscles are concerned are shown in tables 4 and 5.

TABLE 4

Decrease in erythrocyte count after injection of extract of dog's spleen, 10 mgm. per kilogram of body weight

EXPERIMENT NUMBER	INITIAL COUNT	MINIMAL COUNT	AMOUNT OF DECREASE	PERCENTAGE EFFECT	MINIMUM IN	DURATION OF EFFECT
					minutes	*minutes*
21	7,216,000	5,520,000	1,696,000	23.50	30	150
22	6,976,000	5,900,000	1,076,000	15.42	120	150
23	6,656,000	4,561,000	2,095,000	31.49	60	150
24	6,000,000	5,152,000	848,000	14.10	30	120
25	5,952,000	4,592,000	1,360,000	22.84	30	150
Averages	6,560,000	5,145,000	1,415,000	21.47	54	144

TABLE 5

Decrease in erythrocyte count after injection of extract of cat's spleen, 10 mgm. per kilogram of body weight

EXPERIMENT NUMBER	INITIAL COUNT	MINIMAL COUNT	AMOUNT OF DECREASE	PERCENTAGE EFFECT	MINIMUM IN	DURATION OF EFFECT
					minutes	*minutes*
26	6,432,000	5,376,000	1,056,000	16.41	30	90
27	6,136,000	5,400,000	736,000	11.99	30	60
Averages	6,284,000	5,388,000	896,000	14.20	30	75

In both groups of experiments there occurred a decrease in the red corpuscle count similar to that described following the administration of the splenic preparations (Armour and Company). Furthermore, in the experiments with extract of dog's spleen a transient increase in the leucocytes appeared at about the time the red corpuscles were decreased to the greatest extent (table 6). Such a change in the white corpuscle count was not observed, however, after the injection of extract of cat's spleen.

TABLE 6

Increase in the leucocyte count after injection of extract of dog's spleen, 10 mgm. per kilogram of body weight

EXPERIMENT NUMBER	INITIAL COUNT	MAXIMAL COUNT	AMOUNT OF INCREASE	PERCENTAGE EFFECT	MAXIMUM IN
					minutes
21	11,000	11,800	800	7.27	90
22	14,000	19,000	5,000	35.71	30
23	12,000	18,000	6,000	50.00	120
24	12,200	14,000	1,800	14.75	90
25	8,000	10,400	2,400	30.00	30
Averages.....	11,440	14,640	3,200	27.54	72

TABLE 7

Decrease in erythrocyte count after injection of extract of dog's spleen, 20 mgm. per kilogram of body weight

EXPERIMENT NUMBER	INITIAL COUNT	MINIMAL COUNT	AMOUNT OF DECREASE	PERCENTAGE EFFECT	MINIMUM IN	DURATION OF EFFECT
					minutes	*minutes*
28	6,678,000	5,320,000	1,358,000	20.33	90	210
29	7,336,000	5,312,000	2,024,000	27.58	60	150
30	9,632,000	5,888,000	3,744,000	38.87	120	150
31	7,216,000	4,304,000	2,912,000	40.35	120	150
32	6,864,000	4,944,000	1,920,000	27.97	60	150
Averages.	7,545,200	5,153,600	2,391,600	31.02	90	162

TABLE 8

Decrease in erythrocyte count after injection of extract of dog's spleen, 40 mgm. per kilogram of body weight

EXPERIMENT NUMBER	INITIAL COUNT	MINIMAL COUNT	AMOUNT OF DECREASE	PERCENTAGE EFFECT	MINIMUM IN	DURATION OF EFFECT
					minutes	*minutes*
33	5,072,000	2,560,000	2,512,000	49.52	30	120
34	7,644,000	6,272,000	1,372,000	17.94	60	120
35	6,704,000	5,492,000	1,212,000	18.07	90	150
36	6,256,000	4,512,000	1,744,000	27.87	90	150
37	8,720,000	7,344,000	1,376,000	15.77	120	150
Averages.	6,879,200	5,236,000	1,643,200	23.88	74	134

These results establish the presence in the splenic preparations of an agent capable of reducing by some means the number of red corpuscles in the circulating blood. Is this effect quantitative, that is, can it be increased by increasing the dose of the splenic preparation? To determine this point ten additional experiments were carried out. In five of these 20 mgm. of the extract of dog's spleen per kilogram of body weight were injected subcutaneously. In the other five experiments 40 mgm. of the same preparation per kilogram of body weight were injected. The results of these injections on the number of red corpuscles are shown in tables 7 and 8.

The dose of 10 mgm. of extract of dog's spleen per kilogram of body weight decreased the erythrocyte count 21.47 per cent; the dose of 20 mgm. per kilogram decreased the count 31.02 per cent; the dose of 40 mgm. per kilogram decreased the count 23.88 per cent. It would seem from this that the effect is not entirely progressive, but further consideration will be given to this point later.

If the decrease in the number of red corpuscles in the circulating blood is the result of a direct destructive action exerted by the splenic agent on the corpuscles, it should be possible to cause their destruction by bringing them into contact with this agent outside of the body. We tried to determine whether this could be done by the procedure now to be described. Specimens of blood from an ear vessel of a rabbit were obtained simultaneously in two haemacytometer pipettes in the usual manner. The blood in one pipette was then diluted with 0.7 per cent sodium chloride solution while the blood in the other was diluted with 0.7 per cent sodium chloride solution in which was dissolved extract of dog's spleen in definite amount. Several successive drops of diluted blood from each pipette were counted and the counts from each averaged and compared. In order to have a check on any difference in their accuracy the pipettes and counting chambers after thorough cleansing were alternated and new specimens of blood from the same animal secured and counted. Counts made in this way showed close agreement.

According to Meek and Gasser (3) the average volume of the blood in the rabbit is 100 cc. (5 per cent of body weight). Assuming complete absorption after the subcutaneous injections the maximum amount of splenic extract which could be present in the blood at any time would be about 20, 40 or 80 mgm. per 100 cc. of blood for the doses of 10, 20 or 40 mgm. per kilogram of body weight. On this basis we made determinations using dilutions of 20, 40, 80 and 120 mgm. per 100 cc. of 0.7 per cent sodium chloride solution. The results of these observa-

tions are given in table 9. A considerable decrease in the red corpuscle count, which can hardly be explained in any other way than as a direct haemolytic effect of the splenic extract, was obtained. Also the effect was progressive so far as these observations go.

A number of investigators have sought for direct haemolytic action of splenic extracts outside of the body. Nolf (4), Weil (5), Banti (6) and Furno (7) obtained positive results, but Achard, Foix and Salin (8), Widal, Abrami and Brulé (9) and Krumbhaar and Musser (10) were unable to detect any direct haemolytic action of fresh extract of spleen. In these observations the splenic extract was allowed to act upon washed red corpuscles rather than whole, freshly drawn blood. It is probable that washing the corpuscles removes the weaker ones and retains only the more resistant, which the splenic substance is unable to destroy. If the haemolytic substance in the extract of spleen is capable of attack-

TABLE 9

Decrease in erythrocyte count produced by mixing blood with splenic extract outside of the body

NUMBER OF OBSERVATIONS	BLOOD DILUTED WITH NORMAL SALINE	BLOOD DILUTED WITH NORMAL SALINE PLUS SPLENIC EXTRACT	AMOUNT OF SPLENIC EXTRACT PER 100 CC. OF DILUTING FLUID	AMOUNT OF DECREASE	PERCENTAGE DECREASE
			mgm.		
14	6,229,800	5,515,800	20	714,000	11.46
10	6,689,600	5,832,000	40	857,600	12.81
10	6,248,000	5,139,200	80	1,108,800	17.74
6	7,008,000	4,771,000	120	2,237,000	31.92

ing the less resistant corpuscles only we can understand how it was that in the experiments just quoted no reduction of reds was observed, whereas, in our experiments, in which fresh whole blood was used, a marked diminution in their number took place.

Zelenski (11) in 1891 and Danilewski (12) in 1895 were able to cause a marked increase in the number of red corpuscles in the circulating blood and also in the haemoglobin content of the blood following a single intraperitoneal injection of splenic extract. Paton, Gulland and Fowler (13) repeated their experiments without being able to detect any change in the erythrocytes. Silvestri (14) believed that he obtained an increase in the red corpuscle content of the blood in anaemia by the use of injections of splenic extract. Krumbhaar and Musser (10) also were able to bring about an increase in the erythrocyte count and haemoglobin content by the injection of fresh splenic extract. This

increase lasted one or two days and could be re-obtained by repeating the dose.

In each of these cases a blood count was not made until the day following the injection, whereas our counts were begun immediately after the injection and continued at short intervals for several hours. It was at this time the decrease noted by us occurred (see tables 1, 2, 4, 5, 7 and 8 for duration of effect). However, we have noted that when an animal was used for a second experiment after the lapse of a few days its initial red corpuscle count was frequently higher than it had been before any of the extract was administered. The initial count in experiment 21 is 7,216,000. A few days later this rabbit was used again, experiment 30, and then its initial red corpuscle count was 9,632,000; nevertheless a considerable decrease in the erythrocyte count (38.87 per cent) was promptly brought about by the injection of splenic extract.

The bone marrow usually responds to a lowering by hemorrhage and other means of the number of corpuscles in the circulating blood by increased production of new corpuscles, so that in the present instance, where the immediate effect of the splenic agent is a decreased erythrocyte count, it is reasonable to expect subsequently increased production. This compensation might very possibly exceed the loss especially if, as has been claimed by Stradomsky (15), the product of splenic activity, in addition to acting directly to destroy red corpuscles, exerts a stimulating influence on the bone marrow. The results obtained in the group of experiments in which 40 mgm. of splenic extract per kilogram of body weight were injected subcutaneously (table 8) are in accord with this idea of compensatory action on the part of the bone marrow. The table shows that in this series of experiments the percentage decrease and the duration of effect were both less than when the dose was only 20 mgm. per kilogram (table 7). It is possible that the greater the amount of haemolytic agent present the more prompt and more marked is the compensating response. It is more probable that Stradomsky's idea is correct and that the stimulating element of the splenic extract becomes predominant in the larger doses where it is undoubtedly combined with the independent effort of the bone marrow to replace the corpuscles that are being rapidly destroyed.

It is also possible that the effect described may be due to a depression of the haematopoietic activity of the bone marrow by the splenic extract, or to a temporary withdrawal of red corpuscles from the circulation. Such assumptions, however, are unsupported by any evidence of which we are aware.

We believe our results indicate the existence in the spleen of two substances exerting an influence on the number of red corpuscles in the circulating blood—one, a direct haemolytic agent, acting particularly upon the older and less resistant corpuscles; the other, an agent stimulating the production of new corpuscles by the bone marrow to replace the ones destroyed; both operating to keep the mass of corpuscles in circulation of the most efficient quality. Further research in this connection is being undertaken.

CONCLUSIONS

1. The subcutaneous injection of protein-free splenic extract is followed immediately by a decrease in the number of erythrocytes in the circulating blood.
2. The decrease is temporary.
3. The decrease is probably due to a direct haemolytic action of the splenic agent.
4. The decrease is frequently accompanied by a very transient increase in the number of white corpuscles.

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PHYSIOLOGIC CHANGES PRODUCED BY VARIATIONS IN LUNG DISTENTION

II. Efficiency of the Pulmonary Circulation in Overcoming Obstruction

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The experiments on dogs, the results of which are recorded below, are part of a series undertaken to study the relationship between the pulmonary and the systemic blood pressures during distention of the lungs. The endeavor was to distend the lungs while eliminating as many as possible other respiratory factors which might influence blood pressure. To maintain distention of the lungs the entire animal was placed in a plethysmograph from which air could be exhausted and through the wall of which passed a tube allowing tracheal communication with the outside air. Similar tubes permitted exterior connection to recording manometers (1).

With distention of the lungs brought about by this method there was found to exist a surprisingly constant effect upon the carotid blood pressure which could be predicted for the degree of exhaustion of the plethysmograph. With very slight distention of the lungs, there occurred a small rise in blood pressure; with greater distention a marked fall, and with sufficient distention the blood pressure fell to within a few millimeters of the zero of the manometer and carotid pulsations ceased. These phenomena, in view of the fact that the heart continued beating vigorously during their occurrence, indicate a mechanical blocking of the circulation in the blood vessels of the lungs; that this blocking is due to occlusion of the pulmonary capillaries is further evidenced by a rise in pulmonary arterial pressure. In the more extreme degrees the lumen of the pulmonary capillaries was sufficiently reduced to prevent the passage of blood causing a rise in pressure in the pulmonary artery sufficient to distend the right heart.

The degree of negative pressure necessary to obliterate the lumen of the pulmonary vessels may be taken as a measure of the pulmonary arterial blood pressure. To use this method for an actual determination of the pulmonary blood pressure it is necessary merely to determine the minimal negative plethysmographic pressure that will cause disappearance of the pulse in the carotid artery. This degree of pressure can also be estimated by comparing the plethysmographic pressure with the blood pressure and determining the point where the fluctuation in one is equal to the fluctuation in the other. Both conditions indicate zero carotid pressure resulting from obstructed pulmonary capillaries. A more accurate method is to record the intrathoracic pressure for comparison with the negative plethysmographic pressure. In view of the fact that, by this method, the egress of blood from the pulmonary arterial circuit is prevented, the estimate of pulmonary arterial pressure indicates the maximum pressure attainable in this system, and our figures in consequence are considerably above the average usually given for pulmonary arterial blood pressure. They really indicate the maximum efficiency of the right ventricle to overcome resistance. The rise in pulmonary blood pressure will be discussed in a subsequent paper.

Results of experimentation. Since blood pressures and plethysmographic pressures were recorded by mercury manometers, the readings in millimeters of mercury on the unreduced tracings have been multiplied by 2 for correct determinations of pressure changes.

Experimental results are reproduced in figure 1; the sections of the tracing being characteristic parts from a series of thirty-one tests made on a single animal. It is noted that a fall in plethysmographic pressure produced a synchronous fall in blood pressure. This drop in blood pressure was always greater than the corresponding decrease in plethysmographic pressure. Any variation in plethysmographic pressure produced a corresponding change in carotid pressure. When the plethysmographic pressure returns to the atmospheric level, the carotid blood pressure rises to a level higher than that which obtained prior to the experiment; this rise is only transient, however.

The last three sections of the tracing show that there is a decrease in pulse pressure during the distention of the lungs and that there is also an absence of all respiratory waves in the blood pressure curve due to reflex apnoea. A comparison of the pulse pressure in *C* previous to the experimental distention of the lungs with that in *F* shows that there is an increase notwithstanding the fact that the animal had been

given considerable time in which to recover under normal atmospheric conditions from the effects of the previous experiments. This increase in pulse pressure after the performance of several experiments upon an animal is a characteristic to be noted on a large proportion of all tracings as is also the accompanying slow pulse rate. Two tracings are shown in *F* at nearly the same plethysmographic pressures; in the first of these the plethysmographic pressure was held constant after the maximum reduction in pressure, then suddenly released. In the second, the plethysmographic pressure was varied and the carotid pressure shows corresponding variations even at the low pressure level

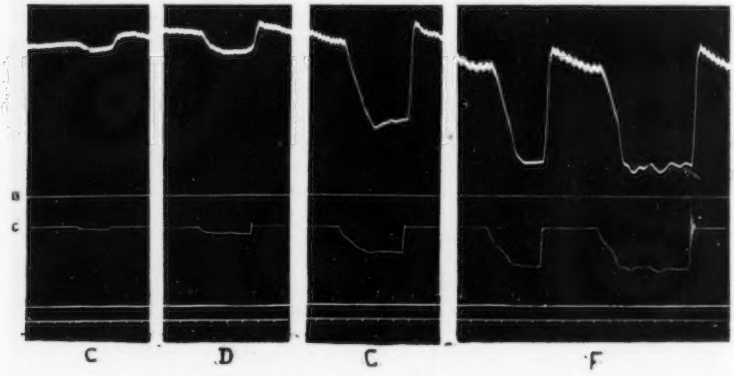

Fig. 1. Experiment of December 4, 1916. Dog of 7¼ kilo. Ether. Time every ten seconds. *A*, Carotid blood pressure recorded by mercury manometer. *B*, Abscissa for carotid blood pressure which is 62 mm. above the time record. *C*, Pressure in plethysmograph recorded by mercury manometer.

at which these variations occurred. It will be noted that in each instance the variations in carotid pressure are greater than the corresponding variations in plethysmographic pressure. So consistently has this occurred with all negative pressures insufficient to completely occlude the pulmonary capillaries, that a fall in carotid pressure equal to the fall in plethysmographic pressure has been taken as an indication of pulmonary occlusion resulting in a depleted systemic arterial circulation and consequent zero arterial pressure.

The results of another procedure are shown in figure 2. In this experiment the pressure in the plethysmograph was gradually decreased to −94 mm. of mercury and subsequently slowly returned to the

atmospheric pressure. Here again it is noted that the fall in carotid pressure is not at all in proportion to the negative pressure in the plethysmograph until the point marked with the arrow is reached. It is at this point that the heart beats failed to record on the carotid pressure tracing, although the heart was vigorously beating at this time. Beyond this point to the point of release of plethysmographic pressure the two curves maintain practically an equal distance by vertical measurement between them, there being a difference of only 2 mm. The maintenance of this constant distance between the two curves, vertically measured, indicates that during this part of the experiment

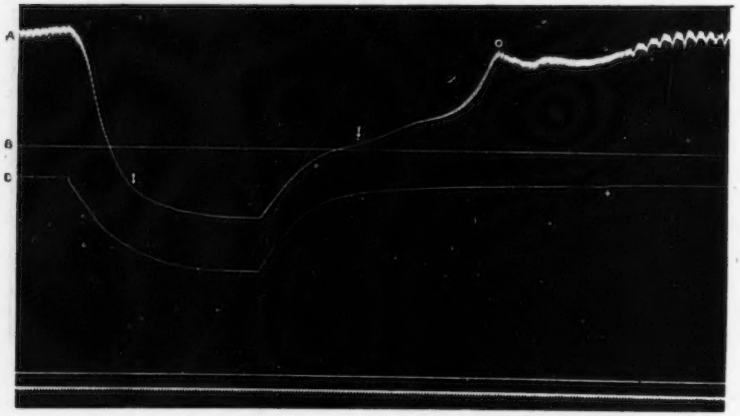

Fig. 2. Experiment of December 1, 1916. Dog about 7.5 kilo. Ether. Time every second. *A*, Carotid blood pressure recorded by mercury manometer. *B*, Abscissa for carotid blood pressure which is 121 mm. above the time record. *C*, Pressure in plethysmograph recorded by mercury manometer. Arrows indicate points of disappearance and reappearance of pulse waves in unreduced tracing. Cross indicates point at which the plethysmographic pressure was finally released. Circle indicates an oscillation common to many tracings.

the fall in carotid pressure is due exclusively to the fall in plethysmographic pressure and not to changes in the animal's blood-pressure mechanism. In other words, the pulmonary circulation is completely blocked and there is a consequent complete failure in the systemic circulation. The degree of negative pressure necessary in this experiment to occlude the pulmonary vessels measured on the original tracing (from the point where the two curves commence to maintain an equal

vertical distance between them) was found to be 40 mm. which, multiplied by 2, equals approximately the maximum pulmonary arterial pressure obtainable in this animal under the favorable conditions of occluded peripheral pulmonary vessels.

On the gradual release of the plethysmographic pressure, reëstablishment of the systemic circulation takes place, and the heart beats again show at the point indicated by the second arrow. It is at this point that the rise in carotid blood pressure becomes greater than the corresponding rise in plethysmographic pressure. The systemic circulation does not become reëstablished until a level of plethysmographic pressure considerably higher than that necessary to deplete it has been obtained. The probable explanation of this lies in the fact that the cardiac muscle has suffered during the period of depleted systemic circulation of which the coronary arteries are a part. The high pulmonary arterial pressure and consequent high intraventricular pressure on the right side of the heart which under these conditions persists even during the ventricular diastole is an added factor preventing the transmission of blood through the right heart muscle itself. A systemic diastolic pressure higher than the right ventricular diastolic pressure is necessary for the circulation of the blood through the muscle of the right heart. The time factor is therefore of importance and to eliminate cardiac failure as a cause in the fall in carotid pressure the duration of an experiment must be short. When the blood pressure approaches its pre-experimental level a variation is seen (circle) which might well be due to the fact that the left heart is no longer flooded with the excess blood which had been retained in the pulmonary arteries by the capillary obstruction due to distention of the lungs. A reflex apnoea is evidenced by the absence of respiratory waves until the plethysmographic pressure again reaches that of the atmospheric pressure at the point marked +.

The results of 49 typical experiments upon five dogs are given in table 1 and the curves plotted from these data shown in figure 3. The graph is so drawn as to show the fall in carotid pressure (ordinates) caused by given changes in plethysmographic pressures (abscissae) which range from zero to a negative pressure of 118 mm. of mercury. The dots indicate plethysmographic pressures and the circles the fall in carotid blood pressure resulting from these negative pressures.

In plotting the fall in carotid pressure due to a given degree of negative plethysmographic pressure, the fact has been taken into consideration that, had the condition of the animal's circulation remained

TABLE 1

	CAROTID PRESSURE BEFORE EXPERIMENT	PLETHYSMOGRAPHIC PRESSURE	CAROTID PRESSURE DURING EXPERIMENT	FALL IN CAROTID PRESSURE CORRECTED	CAROTID PRESSURE AFTER EXPERIMENT	DURATION OF EXPERIMENT IN SECONDS
	113	−1	113	+1	113	56
	113	−2	113	+2	113	20
	121	−4	121	+4	135	30
	143	−5	133	5	123	38
	131	−9	116	6	139	35
	121	−10	90	21	120	30
	148	−12	128	9	167	30
Dog 1.....	135	−16	94	25	140	36
	146	−20	90	36	145	45
	138	−25	75	38	146	42
	141	−31	54	56	128	25
	150	−46	19	85	146	31
	148	−50	−16	114	104	35
	133	−60	−46	119	90	38
	134	−70	−57	121	83	38
	152	−3	146	3	161	20
	155	−6	143	6	168	28
	162	−8	142	12	169	32
	146	−13	116	17	80	40
	142	−14	101	27	166	40
	147	−18	108	21	175	50
	144	−19	90	34	171	48
Dog II.....	150	−22	100	29	174	70
	153	−26	73	54	171	40
	149	−33	56	60	171	42
	134	−36	45	53	150	48
	158	−37	48	73	159	40
	146	−40	38	65	156	40
	136	−41	35	61	147	50
	128	−47	17	64	147	37
	138	−15	101	23	138	28
	130	−17	83	30	113	31
	115	−28	42	45	108	32
	138	−38	25	75	129	35
	104	−39	2	63	120	30
Dog III.....	119	−59	−33	93	140	38
	143	−72	−32	103	156	32
	129	−73	−50	106	143	35
	140	−77	−55	118	145	45
	145	−85	−60	120	119	50
	119	−95	−72	96	116	52

TABLE 1—*Concluded*

	CAROTID PRESSURE BEFORE EXPERIMENT	PLETHYSMOGRAPHIC PRESSURE	CAROTID PRESSURE DURING EXPERIMENT	FALL IN CAROTID PRESSURE CORRECTED	CAROTID PRESSURE AFTER EXPERIMENT	DURATION OF EXPERIMENT IN SECONDS
Dog IV	116	−20	35	52	118	47
	129	−45	−7	91	125	29
	125	−117	−93	101	117	60
	118	−118	−74	74	102	53
Dog V	157	−34	42	81	174	27
	163	−52	8	103	164	11
	166	−90	−68	144	150	18
	150	−102	−88	136	150	18

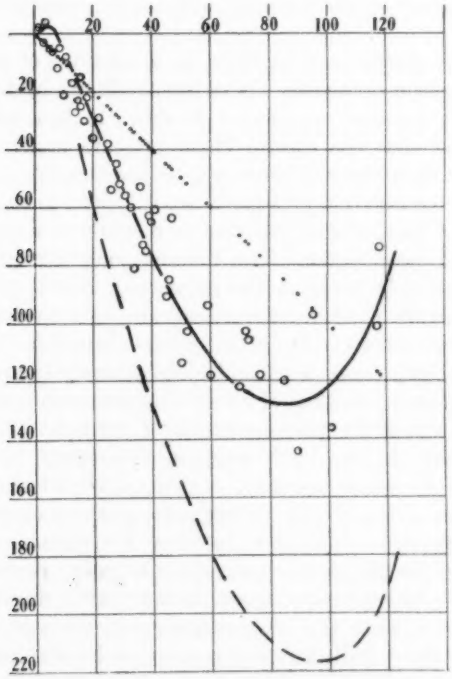

Fig. 3. Composite plotted curves (5 dogs) showing typical behavior of carotid pressure during forty-nine observations at varying degrees of negative plethysmographic pressure. Circles indicate corrected fall in blood pressure. Dots indicate plethysmographic pressure. Ordinates indicate fall in carotid pressures. Abscissae indicate negative plethysmographic pressures. Broken line represents average curve of uncorrected fall in carotid pressure. Unbroken line represents average curve of corrected fall in carotid pressure.

unchanged, the fall in carotid pressure would have equalled the fall in plethysmographic pressure, since both manometers were affected by the same variations within the plethysmograph. The fall in carotid pressure in excess of that in the plethysmograph shows that circulatory changes also occurred within the animal. The difference between the plethysmographic and carotid falls, therefore, indicates the extent of these changes, and it is this difference representing the changes brought about in the animal's circulatory mechanism which has been plotted as the corrected fall in carotid blood pressure.

From these data we have plotted an average curve (unbroken line) for all negative pressures between zero and 118 mm. of mercury. An additional average curve (broken line) of fall in carotid blood pressure has also been drawn to show the fall without the correction made in the first curve.

Both of the average curves serve as a measure of the maximum pulmonary pressure obtainable under the conditions of distended lungs and blocked pulmonary peripheral vessels. It has been shown in previous figures that the carotid blood pressure undergoes a greater variation than does the plethysmographic pressure, provided that the plethysmographic negative pressure is not sufficient to cause a complete blocking of the pulmonary capillaries as a result of which the carotid blood pressure equals zero. The minimal negative pressure which causes a complete blocking in the pulmonary vessels is estimated at the point where the carotid curve commences to show variations equal to instead of in excess of that of the plethysmograph. In the uncorrected (broken line) curve of carotid blood pressure the point at which this curve is parallel to that of the plethysmographic pressure is the index of the maximum pulmonary blood pressure. The degree of negative pressure in the plethysmograph necessary to produce this parallelism is 85 mm. of mercury. In the corrected curve (unbroken line) the index is the degree of negative plethysmographic pressure necessary to produce parallelism between the carotid curve and an abscissa line, since the plethysmographic negative pressure was subtracted from the fall in carotid blood pressure before drawing the curve. It will be noted that the plethysmographic pressure necessary to produce the latter parallelism is the same as in the corrected curve, 85 mm. of mercury.

A further analysis of the corrected fall in carotid blood pressure shows that with very slight negative plethysmographic pressures (zero to 5 mm. of mercury) the average carotid pressure shows a small rise. The

actual fall in intrathoracic pressure is assumed to be less than the fall in plethysmographic pressure, because the latter must overcome the resistance offered by the elasticity of the lungs before it can affect the former. It is for this reason that 5 mm. of mercury is referred to as a slight negative plethysmographic pressure. With a fall of from 5 to 10 mm., on the other hand, the blood pressure shows an actual fall but not to the same extent as the plethysmographic pressure. With still greater negative plethysmographic pressures, 10 mm. to 118 mm., the average blood pressure curve falls to a much greater extent than the plethysmographic, the maximum fall of 128 mm. being attained with a plethysmographic pressure fall of 85 mm. Variations in the fall of carotid pressure are probably due to differences in lung elasticity in different animals and to differences in initial blood pressure.

When the negative plethysmographic pressures are increased below 85 mm., the fall in blood pressure grows proportionately less and finally the blood pressure begins to rise. Emphysema and pneumothorax which were produced at these low pressures offer the most probable explanation of this phenomenon.

GENERAL CONSIDERATIONS

Increase in the capacity of the pulmonary blood vessels brought about by the normal act of inspiration, causes accumulation of blood in the lungs and a corresponding fall in carotid pressure due to the transient retardation of blood flow to the left heart (2). However if at the end of inspiration no further change is made in the thoracic capacity, no further accumulation can occur and a constant stream should again return to the left auricle with a consequent return of blood pressure to its normal level. It therefore is certain that a fall in carotid pressure, such as has been shown to occur in our experiments and lasting as it does through a long period during which the lungs remain evenly distended, cannot be explained as a result of accumulation of blood in the pulmonary vascular area. The amount of blood locked up in the pulmonary vessels under the condition of prolonged distention of the lungs is so small that it may well be neglected as a factor in determining the fall in general blood pressure under our method of experimentation.

The changes in abdominal pressure brought about by distention of the lungs by the method adopted by us, at first would seem to be the opposite of those brought about by natural respiration. One can readily see the abdomen distend under negative pressure and this

creates the impression that the abdominal walls are being acted on by a force pulling them outward and causing a negative pressure in the abdomen which if it occurred would cause engorgement of the visceral vessels and retard return flow of blood to the heart. But, when all the conditions of pressure on the animal are considered, it is seen that the only change in relative pressures that has occurred is in the animal's relation to its intrapulmonic pressure. In other words, there is a pressure in the alveoli of the lungs, greater than the pressure to which the rest of the animal is exposed. The consequent descent of the diaphragm increases relatively the abdominal pressure, as it does in natural respiration. Post-mortem examinations of the abdominal viscera of animals allowed to die under low negative pressure have clearly shown that, far from having an accumulation of blood in these viscera such as would be the case if the abdominal pressure were reduced, there is on the contrary a marked diminution in the amount of blood present in these organs, evidenced by a very distinct blanching. Expansion of gases in the alimentary tract possibly favors this blanching. The fall in carotid blood pressure cannot therefore be explained by the assumption of a bleeding into the abdominal vessels under low pressure.

When artificial respiration was maintained by rhythmically varying the negative pressure in the plethysmograph the muscles remain in apnoeic passivity so that variations in blood flow through them are negligible.

It is conceivable that changes might occur in the position of the crura of the diaphragm which would offer an obstruction to the return of blood to the right heart and thus cause a fall in general arterial blood pressure. Such a condition evidently would tend to congestion of the abdominal viscera, which we have found to be absent. There is furthermore a coincident rise in pulmonary blood pressure, a fact which is not in conformity with obstruction to the entrance of blood into the thorax. We may then eliminate such crural obstruction as a causative factor in the fall of carotid blood pressure.

In many of our experiments the fall in blood pressure occurred without change in heart rate and since apnoea was often caused by the distention of the lungs, the nervous factor of overflow from the respiratory center to the vagus center inhibiting the latter and causing a more rapid heart beat, need not be considered as of influence on the resultant blood pressure. Further indications that change in heart rate brought about by nervous control is not responsible for the fall in blood pressure are the facts that the fall occurs with both vagi cut or

frozen. Changes in heart volume will be discussed in subsequent paragraphs with reference to the supply of blood to the left heart.

The fall in systemic blood pressure is not due to vasodilatation. The initial fall is too rapid to be thus accounted for. Cerebral anemia resulting from prolonged periods of lung distention causes, on the contrary, vasoconstriction, revealed by a gradual and continued rise in the pressure level in the carotid artery and by the blanching of the viscera already alluded to.

The elimination of the above factors as mediating the fall in blood pressure, coupled with positive findings such as a rise in pressure in the pulmonary artery and distention of the right heart indicate clearly that we are dealing simply with an obstruction to the flow of blood through the lung capillaries. The mechanics of this obstruction require further consideration.

Cloetta (3) has expressed the view that

> as the lungs expand two opposing factors act upon the intrapulmonary vessels. When the alveoli first expand, they tend to exert a radical traction upon the small vessels and capillaries and so enlarge them, but as the enlargement proceeds and the alveoli acquire a polygonal shape, they tend to compress the intrapulmonic vessels. Furthermore, as the lungs enlarge, they necessarily cause a linear extension of the blood vessels and thereby further reduce their calibre. From this it appears that a moderate distention of the lungs causes a diminished resistance, but an extreme distention an augmented resistance.

In the case of Cloetta's method of lung distention in which the lungs alone are contained in the plethysmograph, the heart and large blood vessels are not subjected to variations in pressure synchronously with the lungs as occurs in normal respiration, and as de Jager (4) has pointed out, the capacity of the large extra pulmonary vessels may increase when the surrounding pressure becomes more negative. It is noteworthy that by subjecting the entire animal to negative pressure as we have done, in contrast to the method of Cloetta, the aspiratory effect due to enlargement of the blood vessels by distention of the thoracic cavity is negligible, if we disregard the fact that the thoracic and abdominal cavities are more expansible than the solid tissues of the body.

Concerning the rise in carotid blood pressure with very moderate negative pressure in the plethysmograph and the fall with greater pressures, our findings with a somewhat different method are in conformity with those of Cloetta. But his explanation of these phenomena based on the hypothesis of polygonally shaped alveoli making

pressure on the pulmonary blood vessels, is open to objections which seem of sufficient weight to exclude it. Even if the blood vessels of the lungs could be as nicely placed in reference to the surrounding cells, as assumed by Cloetta (fig. 4) a fractional part of the elastic force of the distended air sacs might be exerted on the walls of the blood vessels only by the assumption of a shape and deformity such as is

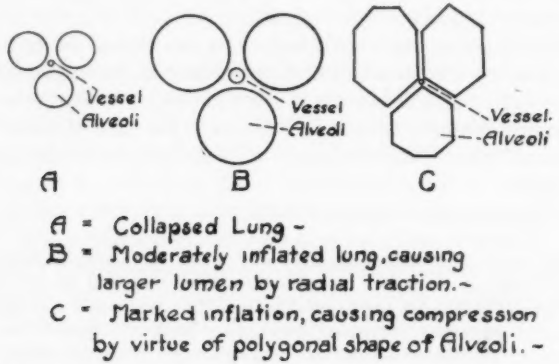

Fig. 4. Diagrams illustrating the effect of lung inflation on the alveoli and lumen of the intrapulmonary vessels (after Cloetta).

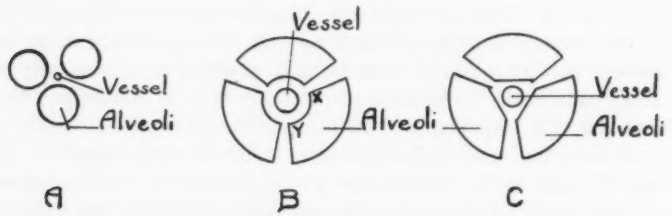

Fig. 5. Diagrams illustrating effect on the caliber of the pulmonary blood vessels produced by change in shape of the alevoli.

illustrated in figure 5, *B*. Should the walls of the alveoli be sufficiently movable for the elastic pull due to their distention to be equally exerted at all points in their circumference in directions tangent to the surfaces at those points, it would follow that the effect of this tangential pull would be to straighten the line *xy* (fig. 5) thereby compressing the blood vessel; but such a resultant pressure could be obtained only when a

part of the curvature of the alveolus was concave with reference to the blood vessel, and never with polygonally shaped cells in which the resultant of the elastic pull inward for converse reasons would be accentuated at the angles.

While stretching of the capillaries of the lungs in their long diameter causing a diminution in their caliber and obstruction to blood flow is undoubtedly a factor in the fall in carotid pressure, it does not seem probable that it can result in complete occlusion of the vessels. The extent to which capillary diminution can be brought about by this means is under investigation.

Of the factors that might be the cause of the fall in carotid blood pressure, there remains the relative increase in intrapulmonic air pressure, which apparently is the essential cause.

In considering the pressure of the intrapulmonic air in its effect on the pulmonary vessels, under the conditions of our experiments, it must be borne in mind that while there is no change in the relation of the intrapulmonic air to the atmospheric air while the lungs are distended, yet there is a considerable change in the intrapulmonic air with reference to the animal subjected to the negative pressure. If an animal be subjected to a given negative pressure while his intrapulmonic air is still in free communication with air at atmospheric pressure it is evident that distention of the air sacs commences and continues until this elasticity plus the elastic resistance offered to increase in the capacity of the thorax equals the increased intrapulmonic pressure. The factor responsible for the distention of the lungs is the difference between the intrapulmonic pressure and the external pressure on the animal; it would be indifferent whether this distending pressure is obtained by diminishing the external pressure on the animal or by increasing the intrapulmonic pressure. It is furthermore clear that under either of the conditions stated above, vessels situated between the alveoli, as the pulmonary capillaries are, must likewise be subjected to the distended pressure. In other words, the pulmonary capillaries lying between adjacent alveoli are compressed between two opposing forces when the intrapulmonic air pressure is increased.

It would follow from this that with varying degrees of negative pressure in the plethysmograph corresponding degrees of pressure exerted in the pulmonary vessels impede proportionately the pulmonary circulation. The fall in carotid pressure is due solely to this interference with the return of blood to the left heart. The total failure of systemic circulation is due to the fact that even the maximal pressure

obtainable in the pulmonary arteries is not sufficient to overcome an external resistance of more than 85 mm. of mercury. This maximal pressure, attained during the period of complete occlusion of the pulmonary vessels, while indicating relative inefficiency in the muscles of the right ventricle compared to those of the left side of the heart, yet is considerably greater than is usually given for average pulmonary arterial blood pressure.

We are indebted to Prof. W. E. Garrey for helpful suggestions and criticisms.

CONCLUSIONS

1. When the lungs are artificially distended by diminishing plethysmographic pressure: *a*, with slight distention there is an inconspicuous rise in carotid blood pressure due to the freer passage of blood to the left heart; *b*, a moderate distention always causes a fall in carotid pressure, the greater the negative pressure in the plethysmograph the greater is the reduction of the carotid pressure, the fall in carotid pressure always being greater than the causative negative pressure in the plethysmograph; *c*, with full distention there occurs a fall in carotid pressure approximately to zero; *d*, with over-distention, causing emphysema and pneumothorax, there is a slighter fall than that produced by less distention.

2. The fall in carotid blood pressure caused by moderately diminished plethysmographic pressure is due to failure in the supply of blood to the left heart.

3. This failure of blood supply to the left heart is due to pressure exerted almost directly upon the small pulmonary vessels.

4. The measure of the degrees of pressure necessary to occlude the vessels can be taken as an index of the pressure in the pulmonary arteries.

5. During a short period of occlusion of the pulmonary capillaries, the blood in the pulmonary arteries may attain a pressure of 85 mm. of mercury.

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EXPERIMENTS ON THE PATHOLOGICAL PHYSIOLOGY OF ACUTE PHOSGENE POISONING

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Phosgene is one of the lethal asphyxiant war gases. If its effects on the animal organism were to be described in one phase, it would be by the words "pulmonary edema." A study of phosgene poisoning is therefore of more than passing medical interest. The work here reported was done by the Wisconsin Section of the Medical Division of the Chemical Warfare Service in an attempt to get a physiological background for proposed forms of treatment.

The general symptoms of gas poisoning have recently been well described by Underhill (1) and since our experience on this point is identical with his, such description need not be repeated here. Underhill's division of phosgene poisoning into three stages is also in accord with our findings, and although arbitrary in certain cases, as all such classifications must be, it is of distinct advantage in locating the various physiological disturbances and in gaining an insight into the condition as a whole.

Our work has consisted in following as closely as possible various physiological reactions throughout the course of fatal phosgene poisonings. We have directed particular attention to the following: arterial blood pressure, venous blood pressure, pulse rate, hemoglobin

[1] The work here reported was done in the Medical School Laboratories of the University of Wisconsin. Problems of gas warfare were initiated under the Bureau of Mines and continued under the Wisconsin Section, Medical Division, Chemical Warfare Service. In addition to the authors, who directed and carried on the major part of this research, especial mention should be made of the services of Dr. S. A. Amberg of the Otho S. A. Sprague Institute, and Mr. J. Y. Malone of the Wisconsin Section, who greatly aided in many phases of the work. Among others associated in a general way with the physiological studies carried on at Wisconsin, there should be mentioned Dr. H. S. Gasser of the Washington University Medical School, and Dr. B. H. Schlomovitz, Dr. P. F. Clark and Mr. L. F. Richdorf of the Wisconsin Section.

determinations, blood volume, red blood cell counts, histological examination of the lungs, x-ray studies of heart and lungs, alkaline reserve, respiratory rate and volume of respiratory ventilation.

Methods. Dogs were used throughout these studies. The animals were subjected for thirty minutes to air containing 80 to 100 parts per million of phosgene. This was sufficient, with rare exceptions, to produce death in the first twenty-four hours. The technique of this gas administration was one gradually evolved at the various Chemical Warfare laboratories. It consisted of placing the dogs in a one-hundred-liter air-tight glass box through which air was drawn at the rate of one hundred liters per minute. The phosgene cylinder was connected to the air inlet tube, the gas outflow being regulated by a needle valve and the rate roughly determined by a flowmeter. From the gassing chamber itself air was drawn in order to determine by chemical analysis the exact concentration of phosgene to which the animals were subjected.

All animals on which it was necessary to make incisions were morphinized either before or immediately after being gassed. A series of morphine controls had been carefully studied and we are sure none of the effects to be described are attributable to morphine. In order to make observations rapidly and frequently the animals were kept tied to operating boards. Since no pressure was exerted anywhere except by the cords on the limbs, and the respiratory passages were entirely unobstructed, this restraint seemed unobjectionable. The animals lay quietly and comfortably until the usual asphyxial stimulations occurred shortly before death.

Arterial blood pressure. By attaching a mercury manometer to the femoral artery, arterial blood pressure records were made in the usual way at half-hour intervals. The general course of the blood pressure in a typical case of phosgene poisoning may be seen at a glance by referring to figure 1. This composite curve, in common with those that are to follow, was made by dividing each of the experiments in the series into ten equal periods. Our animals lived an average of sixteen hours after gassing. Each of the ten intervals therefore represents on the average a little over one and a half hour. The data for the same period in all the experiments were averaged and the results plotted as a composite curve.

A few animals showed a slight fall of blood pressure after being taken from the gassing chamber. In most cases this was insignificant and it did not lower the composite curve during the first period. As

a rule the blood pressure gradually rose during the first half of the experiment increasing some 10 per cent above the normal. Beginning with the sixth period it began to fall slowly, reaching normal at the beginning of the eighth interval. Once having passed the normal the decline became extremely rapid and continued without intermission until the death of the animal.

This break in blood pressure which occurred at the eighth period was an extremely striking event in all the animals studied. It made

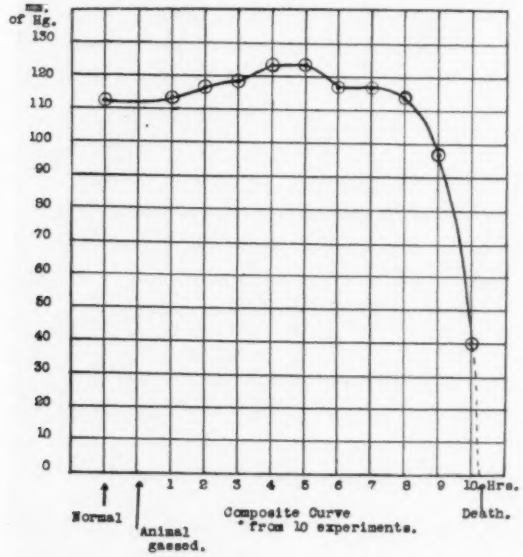

Fig. 1. A composite curve of arterial blood pressure in acute phosgene poisoning. The abscissa indicates the ten periods into which each experiment has been divided.

possible a very accurate prediction as to how much longer the animal would survive. Until its significance was fully appreciated, many of the animals died before final observations could be made.

Figure 2 is a reproduction of the actual records from a typical experiment. The points just mentioned may be noted. The first record at 11:10 a.m. was taken shortly after gassing. The pressure then rose gradually for more than six hours. At 12:10 a.m., thirteen hours after gassing, the pressure was still normal, though falling. One hour and twenty minutes later the animal was dead.

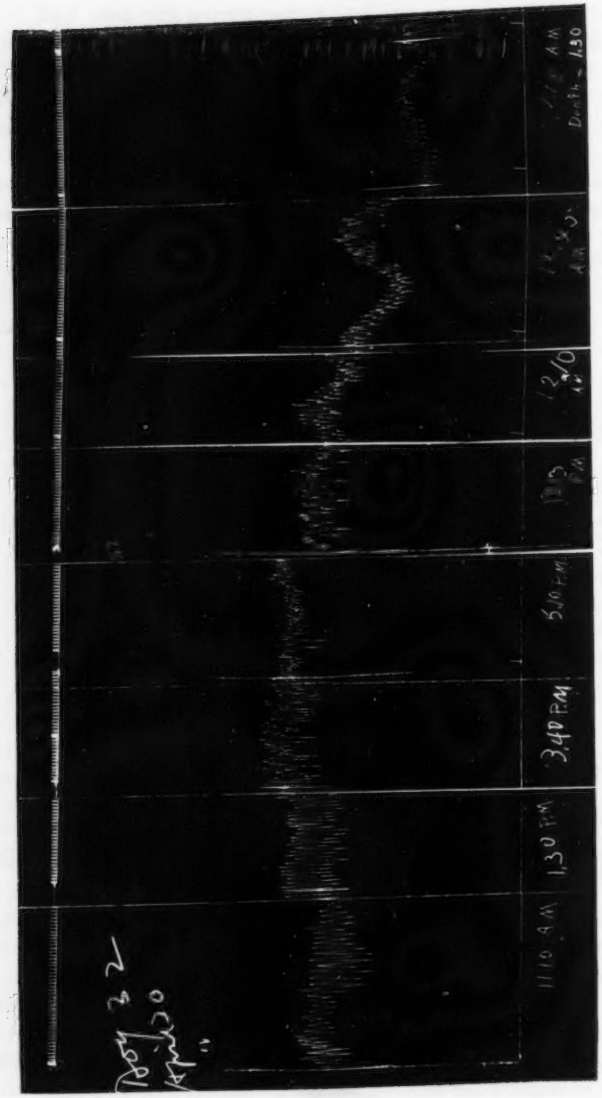

Fig. 2. Arterial blood pressure record from a case of acute phosgene poisoning.

One need not attempt to interpret the arterial blood pressure curve of acute phosgene poisoning until all the other data have been presented. Its resemblance to an asphyxial vasoconstriction is however obvious. That the rise may be due to vasoconstriction and the fall in part to paralysis of the vasomotor center is also suggested by the large vasomotor waves which so often occurred late in the blood pressure tracings (see fig. 2 at 12:45 a.m.).

Venous blood pressure. In the preceding series of ten experiments venous pressure observations were made simultaneously with the arterial. These were secured by inserting a sound into the femoral vein. The sound was connected to a manometer and a reservoir of Ringer's solution. The pressure in the system was raised above what the reading was likely to be. On removing a clip the blood pressure was balanced against that of the fluid in the manometer. The reading could be made before there was any tendency to clot and the fluid added to the blood stream at each observation was negligible.

Venous pressure was found to be rather variable, conforming on the whole however to what one might expect from the arterial. During the long period of increased arterial pressure, venous pressure was either normal or slightly below. In the terminal stages, however, it often rose markedly. In two of the ten experiments there was a noticeable increase in venous pressure immediately after gassing. These were very severe cases, death occurring within nine hours. It would seem probable that in these animals the initial injury to the lungs was so great that the pulmonary circulation was obstructed and venous pressure therefore forced to rise.

Pulse rate. Shortly after gassing the pulse rate fell in practically all cases. This occurred in animals morphinized before gassing as well as in those that received none of the drug. It was therefore an expression of the action of the poison itself. The decrease in rate brought the pulse from an average of 95 to 70 beats per minute. By the time the experiment was half over the heart rate had returned to normal and following this it very markedly accelerated. Figure 3 presents a composite curve from fourteen experiments in which the pulse rate was carefully followed. The final determination of the curve is the average of the highest rates obtained in the tenth period. As death became imminent the heart rate became irregular and the rate then, of course, decreased. Electrocardiograms taken at this time showed various kinds of blocks, dropped beats and extra systoles, features which characterize most records taken during death by asphyxiation.

The pulse rate offered an excellent means of following the condition of the animal. Two of the dogs in one of our series recovered. The pulse curves from these animals were of special interest. There was the initial fall and the subsequent rise, but the latter never exceeded 120 beats per minute. A large number of observations have confirmed our opinion that a fatal outcome is to be expected if the heart rate continues to rise above 125 or 130 beats.

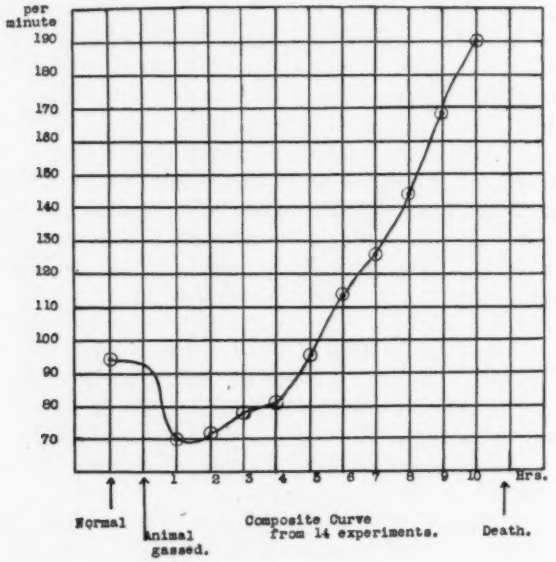

Fig. 3. A composite curve showing the average heart rate in acute phosgene poisoning.

Hemoglobin determinations. It was soon realized that there were very significant changes in the hemoglobin content of the blood in dogs suffering with phosgene poisoning. At death the blood was viscous, even tarry in consistency, and the hemoglobin readings showed an almost unbelievable concentration. Underhill first pointed out that this stage of concentration was preceded by an initial one in which the hemoglobin content of the blood was decreased.

The characteristic hemoglobin changes during the course of the poisoning may be seen in figure 4. In an average experiment the hemoglobin

readings were below normal during the first four periods of the experiment. This constitutes Underhill's first stage of phosgene poisoning. Concentration then began and in the period preceding death the hemoglobin readings averaged over 150 per cent. The periods of concentration make up Underhill's second stage.

The maximum decrease in hemoglobin occurred anywhere during the first four periods, in other words, during the first five or six hours of the experiment. Since the maximum decrease did not occur in the same period for all the experiments, the composite curve does not show the lowest limit reached in hemoglobin concentration. In the sixteen cases reported, it actually averaged 11 per cent, that is, a hemoglobin reading of 89. The lowest reading noted was 85, although in treatment

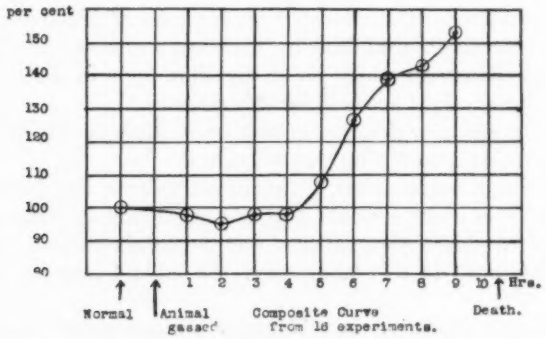

Fig. 4. A composite curve of hemoglobin concentration in acute phosgene poisoning.

series not here reported we have had readings as low as 80 and one of 78. The maximum decrease in our experience lasted a very brief time and often there was difficulty in making desired observations at exactly the proper moment.

The degree of subsequent hemoglobin concentration has always borne a definite relation to the severity of the poisoning and made possible a rather accurate prognosis. One hundred and twenty-five per cent may be said to represent a critical point. Any animal exceeding this figure was pretty sure to die in the course of the next five or six hours. If an animal was not gassed enough to reach this concentration, or if by any means a 125 per cent concentration could be prevented, there was an excellent chance for recovery.

Hemoglobin readings are usually interpreted in terms of the fluid content of the blood, or blood volume. If plasma or water has left the blood stream there is of course a concentration of hemoglobin, and if fluid has entered the blood stream from the tissues or elsewhere there is naturally a lowered hemoglobin content. While this is the general rule, there may be exceptions. Hemolysis or stagnation of red blood cells at any point might very greatly modify hemoglobin determinations and yet the blood volume would be entirely unchanged.

The natural interpretation of the hemoglobin curve for phosgene poisoning would be that in stage 1 there is an increase of blood volume and in stage 2 a marked decrease. That there is a real decrease in stage 2 is borne out by the fact that the lungs are now full of fluid which must, of course, have come from the blood. There is, however, no equally obvious explanation for an increase of blood volume in stage 1. Underhill (1) also finds in this stage a decrease in the blood chlorids, but the excess which he at the time finds in the urine and fluid of the lungs might account for this decrease.

In order if possible to clear the matter up we have made direct blood volume determinations during stages 1 and 2.

Blood volume determinations. The blood volume has been determined directly in eight animals during stage 1 and in three animals during stage 2. The technique used was the acacia method (2) which has been developed in this laboratory. In table 1 may be seen the results.

The data presented give no evidence of a blood volume increase in stage 1. In a large series of normal dogs the volume has frequently been 10 and 11 per cent of body weight with an average of 9.7 per cent. The eight animals here investigated averaged then within 2 per cent of normal. Furthermore of the three animals having the greatest hemoglobin dilutions, only one had a volume above the average.

In stage 2 only three determinations were made and unfortunately no hemoglobin readings were made simultaneously. Red blood cell counts, which have always paralleled the hemoglobin readings, indicated however that the latter were in the neighborhood of 135 per cent. Since the technique required the removal of 20 cc. of blood, the determinations were made some time before the anticipated death of the animal. The figures particularly the first and third show that these animals had a marked decrease in blood volume. Even 8.1 per cent body weight is a lower blood volume than we have ever found in a normal dog.

Stage 1 of phosgene poisoning, on the grounds of blood volume data, we believe to represent an actual decrease in the total hemoglobin content of the blood, a point which will be discussed later under the heading histological examination of the lungs.

In stage 2 there can be no doubt that the blood volume is actually greatly decreased.

Red blood cell count. Little need be said concerning the red blood cell counts other than that they uniformly paralleled the hemoglobin

TABLE 1

Showing blood volumes in phosgene poisoning

WEIGHT OF ANIMALS	Hb. AT TIME OF DETERMINATION	BLOOD VOLUME IN PER CENT OF BODY WEIGHT
	Stage 1	
kgm.	*per cent*	
9.80	88.4	8.7
9.58	90.9	9.6
9.14	91.0	11.1
9.82	94.4	10.8
8.48	96.0	8.7
8.42	92.0	9.5
7.22	83.5	11.2
5.70	84.5	9.9
Average		9.9
	Stage 2	
14.00		6.5
13.8		8.1
13.5		7.5
Average		7.3

determinations. A composite curve from nine experiments may be seen in figure 5. As in the hemoglobin curve, stages 1 and 2 are evident and they occupy the same relative positions.

Histological examination of the lungs. Dr. W. S. Miller of the Department of Anatomy has kindly made a careful histological examination of lung tissue in a series of poisoned dogs. He has used his special methods for fixing and staining pulmonary tissues rather than the routine technique of general pathology. These examinations show that the injury from phosgene is almost exclusively in the lower

respiratory passages. There is constriction or spasm of the small bronchioles with the accompanying atelectasis and emphysema. There is edema of the connective tissue. The alveoli are irregular, their membranes injured, and in many cases they contain exudate. Very important from our point of view is the extensive clogging of the capillaries with red blood cells. Even small veins are solidly plugged. In many cases these masses in the veins have shrunk slightly and the surrounding clear areas are filled with serum. In figure 6 may be seen a photomicrograph illustrating the points just mentioned. That these changes in the lungs are not post-mortem is substantiated by the fact that they are characteristic of all the early

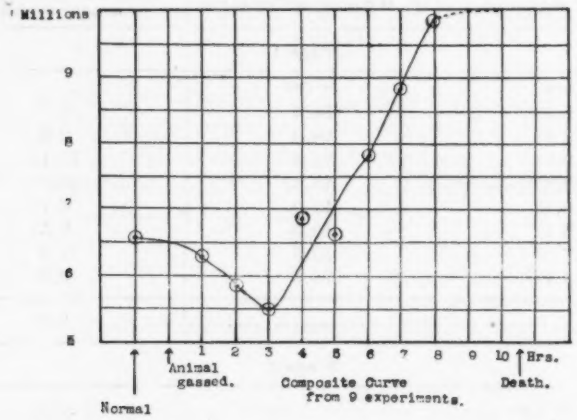

Fig. 5. Red blood cell counts in acute phosgene poisoning.

stages of poisoning and that they were found after every attempt to avoid post-mortem clot.

Furthermore, if phosgene in a dilution as great as 1 to 20,000, that is 0.222 milligrams per liter, be bubbled through a 2 per cent suspension of defibrinated dog's blood, there is in twenty minutes a marked agglomeration of the red corpuscles. This is direct evidence that the gas has the power of doing just what the histological picture shows has been done.

In slightly later stages than the one figured, there is evidence that compensatory paths are being opened up for the blood stream. Capillaries which were not plugged are widened and others have been dilated sufficiently to allow a flow of fluid around the obstructions.

The importance of these histological findings on the physiological conception of phosgene poisoning is at once apparent. The plugged capillaries and veins must at first greatly increase pulmonary resistance and the work of the right heart. Later there is relief by the develop-

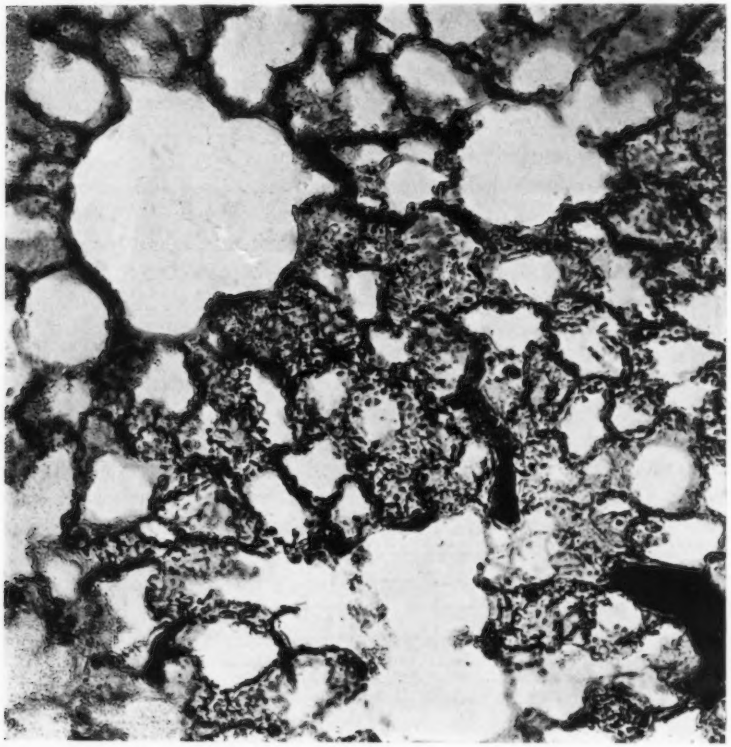

Fig. 6. A photomicrograph from the lower left lobe of a dog's lung, killed one hour and forty minutes after being gassed. Especially to be noted are the capillaries gorged with red blood cells, the alveolar exudate and the pulmonary vein in the lower right hand corner which is plugged with a mass of blood.

ment of compensatory passages. Just how the heart reacts to this we shall see in the following section. Furthermore the injuries to the alveolar walls must decrease the exchange of gases between the blood and the alveolar air.

Heart size. In a large series of experiments stereoscopic examinations and x-ray photographs of the thorax have been made at frequent intervals. At first the outline of the heart was sketched in with a grease pencil on the glass cover of the fluoroscope. Later stereoscopic plates were made and these, of course, proved much more reliable than the former method. To be sure that the animal was in the same position for each photograph, a lead cross was sewed to the chest and the center of this brought under a plumb bob at each exposure. Exposures were always made during the same phase of respiration, preferably inspiration, and the flashes were long enough to insure that it was the diastolic size of the heart which was secured.

These observations have shown two interesting and important changes in heart size during acute phosgene poisoning. There was first, immediately after removal from the gas chamber an increase in the size of the heart which varied considerably in degree, but was always associated with a relative enlargement of the right auricle and ventricle. This condition persisted for several hours and might even increase for an hour or more.

By the beginning of the fourth period, assuming that the experiment had been divided into ten equal intervals, a second change appeared which was a gradual reduction in heart size. This seemed to appear first in the left ventricle, but soon the whole heart became distinctly smaller. It assumed a pendular shape which was apparently identical with that following severe hemorrhage, as determined in control experiments. This decrease in size continued during the development of the extensive pulmonary edema. During the period of asphyxial death the heart began again to enlarge, particularly the right side, and after the death plates invariably showed a dilated heart with relative increase of the right side.

Figure 7 illustrates strikingly the stages of increase and decrease in heart size. The area of the normal heart shadow as determined by the planimeter was 51.2 sq. cm. Thirty-nine minutes after gassing, the area had increased to 55.4 sq. cm. Eleven hours and fifty-three minutes after exposure the heart had decreased to 44.5 sq. cm. This was at a time when the pulmonary edema had become very marked.

Changes in the lungs. The condition of the lungs was determined in a large number of animals by means of physical, fluoroscopic and stereoscopic x-ray examinations. Immediately after removal from the gas chamber there was noted a diffuse clouding of the lungs usually most marked in the middle and upper lobes but sometimes involving

the lower lobes also. This was accompanied by an increase in density and number of the streaky shadows cast by the larger bronchi and vessels at the roots of the lungs.

For a few hours the cloudy appearance of the lungs generally increased slowly without however being associated with any constant physical signs. Occasionally, transitory fine dry crackling râles were heard during expiration, and occasionally there was a slight roughening of the normal respiratory sounds.

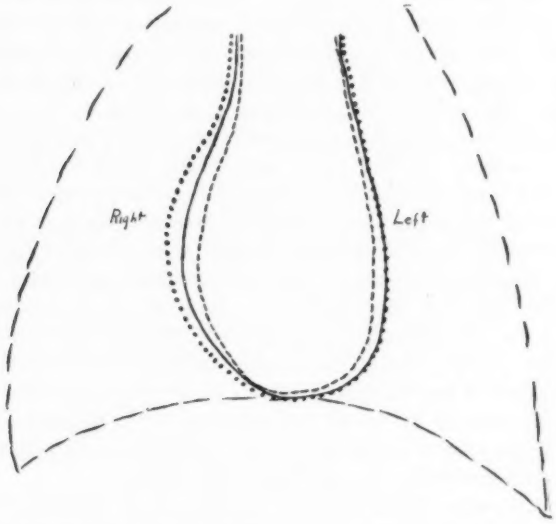

Fig. 7. Superimposed outlines of three x-ray photographs taken at intervals during phosgene poisoning to show changes in shape of the heart. The solid line indicates the normal. The dotted line shows the right-sided dilatation thirty-nine minutes after gassing. The broken line is from a photograph taken eleven hours and fifty-three minutes after exposure to the gas. The heart had then become pendular in shape and much reduced in size.

After these few hours the lungs often appeared somewhat clearer. This improvement, however, was transient and gave way during the latter third of the experiment to a streaky, mottled appearance, at first marked near the roots of the lungs but later involving the whole of both lungs. Often the heart outline was in part lost or obscured. It was at this time that numerous râles of all varieties made their appearance, medium moist predominating. These were heard in both

inspiration and expiration. In brief, the clinical signs were now those of extensive pulmonary edema and passive congestion.

In the later stages there was frequently an impairment of the percussion notes, most evident in the pendent portions, and an extension of deep cardiac dullness especially on the right side. X-ray plates showed, however, that this was not due to cardiac enlargement but probably to better transmission of heart dullness by the edematous lung.

There are thus three more or less distinct changes to be made out by means of the x-ray in the lungs of animals fatally poisoned with phosgene. First a diffuse cloudiness due probably to the initial epithelial injury and to the agglomeration of the corpuscles in the capillaries. Second, an improvement, a decrease in the cloudiness, accounted for by a reopening of many of the capillary passages. Third, a marked increase in the density and extent of all shadows cast by bronchi and blood vessels and an extension of the mottled appearance to all parts of the lungs. It is worthy of mention that it is only during this latter stage that the classical clinical signs of pulmonary edema develop. The x-ray has proved a much more delicate method of following lung lesions than the older methods of percussion and auscultation.

Respiratory rate and pulmonary aeration. One of the earliest results of phosgene gassing was an increase in the respiratory rate. By the end of the fourth period, that is stage 1, the rate had usually increased from an average of 30 to 45 per minute. This increase continued through stage 2 until the death period itself, when the respirations of course became irregular and gradually less rapid.

Respiratory rate, particularly in the dog where there may be much panting, gives a poor idea of the amount of air actually passing in and out of the lungs. To secure such data a series of five experiments was run in which the dogs were placed in an air-tight rigid chamber that enclosed the entire animal except the head. An inflated rubber collar secured an air-tight fit around the neck. The box was connected by tubing to a piston recorder which not only made a record of the respiratory rate but on calibration gave an accurate measure of the air passing in and out of the lungs.

The data thus secured, as shown in table 2, indicate in all cases a final marked increase in pulmonary aeration. In all cases the amount of air respired was at least doubled in the latter periods of the poisoning. Even after the break in arterial pressure when respirations often slowed down, the increase in aeration was maintained. In three cases

the amount respired immediately after gassing was definitely lower than normal.

Temperature. The only constant change in temperature was a gradual fall as blood concentration increased and death became imminent. This amounted to as much as 2 degrees in many cases. Very frequently there was a slight initial rise in temperature during stage 1.

Alkaline reserve. Our studies on alkaline reserve are very incomplete. Determinations taken at irregular intervals in eleven experiments seem however to justify the statement that there is no change of particular significance until the latter periods of the experiment. At about the time blood pressure falls so markedly there is a decided decrease in the alkaline reserve. The tissues at this time are undoubtedly suffering from oxygen want and the decrease in carbonate is due to the formation of fixed acids.

TABLE 2

Showing pulmonary aeration during phosgene poisoning. Aeration in cc. per minute

EXPERIMENT NUMBER	NORMAL	IMMEDIATELY AFTER GASSING	ABOUT ONE HOUR BEFORE DEATH
34	1360	420	3000
35	1265	850	2501
36	950	1540	2080
37	1175	1650	2660
38	1370	1060	3080

DISCUSSION

The various pathological physiological studies just reported are of interest chiefly in giving us a conception of phosgene poisoning as a whole. In fatal cases, and our studies were made on such, two rather well-marked stages are apparent. The first of these is characterized by the nervous reflexes due to the irritation of the gas in the respiratory passages and by the direct chemical action of the gas or its decomposition products on the blood. The second stage is characterized by well-developed pulmonary edema and its natural consequences. The whole subject indeed might well be termed a study of pulmonary edema induced by phosgene.

The first effect of the gas is to injure the linings of the deep respiratory passages. Spasm of bronchiole musculature is evidence of the

stimulation produced by the fumes. As a result of this we have a reflex cardiac inhibition, very characteristic of many stimulations of the respiratory surfaces. The composite curve of heart rates shows a decrease during the first half of the poisoning. Another reflex from the same cause is a vasomotor one which brings about peripheral constriction with a rise in blood pressure. The x-ray gives evidence of pulmonary injury at this time although physical signs are usually entirely absent. More important than these nervous phenomena, however, is the direct action of the gas on the blood in the pulmonary capillaries. Here the red corpuscles are agglomerated into masses which largely fill and block the capillary passages. Bubbling gas through blood shows that it may have just this effect. The results of this plugging of the capillaries are twofold. In the first place pulmonary resistance is increased and a load thrown upon the right heart. Evidence of this is seen in the right cardiac dilatation found in the x-ray plates. A second result is the removal of red cells from the circulation which results in a decreased hemoglobin content of the blood. This first stage of phosgene poisoning, as shown by Underhill, is most easily determined by following the hemoglobin and it may be spoken of as the stage of decreased hemoglobin concentration.

Underhill (1) has explained the decreased hemoglobin concentration on the basis of blood dilution by body fluids. Just how or why blood volume should be increased at this time is not clear. That this interpretation is probably not sufficient we believe to be shown by direct determinations which indicate no increase in blood volume, and by the histological examinations which show the red cells agglomerated in the capillaries.

Long before the end of the first stage pulmonary edema is under way. The direct cause of this is undoubtedly the increased permeability of alveolar and capillary walls due to direct injury from the gaseous fumes. The increased pulmonary blood pressure resulting from the capillary plugging greatly favors the condition.

The second stage of acute phosgene poisoning is characterized by rapid development of the pulmonary edema with all its physical signs. This results in a decreased blood volume and increased hemoglobin concentration and a smaller diastolic size of the heart. The essential thing is the greatly reduced blood volume, which is almost entirely accounted for by the increased fluid in the lungs.

Death under such conditions may obviously be accounted for in either one of two ways. The edematous condition of the lungs may

interfere with the gaseous exchanges to such an extent that the animal asphyxiates, or the blood volume may be so reduced that even though the hemoglobin is oxygenated there is not enough fluid to secure its proper distribution to and circulation in the tissues. So far as the tissues themselves are concerned the result is the same. They die of oxygen starvation. In our opinion death is due to a combination of the two causes. Our belief is based on a series of experiments in which gassed animals were immediately placed in chambers containing 40 to 60 per cent oxygen. The CO_2 content was of course kept within physiological limits and oxygen supplied automatically so as to keep the amount constant. The majority of these animals lived from 48 to 72 hours instead of the average 16 and seemed to be recovering. On being released the usual occurrence was for each dog to walk across the room and fall into an asphyxial convulsion which quickly terminated in death. Several of the animals were hurried back into the oxygen chamber and resuscitated. These animals were edematous with reduced blood volumes, but in an atmosphere of 40 per cent oxygen life was preserved, one is tempted to believe because of complete hemoglobin saturation and physical absorption of oxygen. That decreased blood volume is a cardinal part of the syndrome, and possibly by far the most important part, need not be questioned.

We do not at this time wish to discuss the treatment of phosgene poisoning further than to point out the suggestions naturally arising from our work. In stage 1 bleeding may serve to relieve the right heart and reduce pulmonic pressure. The heart would thus be in better shape for succeeding emergencies and the edema would develop less rapidly. This treatment alone might in many cases tide the animal over and make possible its recovery.

In stage 2 oxygen is of course suggested. Administration of oxygen, although of value, must be looked upon as more or less palliative. It can neither restore alveolar epithelium nor reduce the edema. Wilson and Goldschmidt (3) have recently shown in chloropicrin poisoning that oxygen administration does not prevent concentration of the blood. It may, however, make life possible until the body can take care of itself. Any treatment which would permanently increase blood volume would of course be fundamental.

SUMMARY

1. A study of the pathological physiology of acute phosgene poisoning shows a well-marked succession of events which finally results in typical pulmonary edema. The microscope and the x-ray both show an early injury to the linings of the deep respiratory passages. Irritation from this results in a certain amount of reflex cardiac inhibition and vasoconstriction. Coincident with these changes there is a direct action of the gas on the red blood cells, which causes them to agglomerate and obstruct the pulmonary capillaries. The removal of red blood cells from the active circulation in this way results in a decreased hemoglobin percentage. The plugging of the capillaries throws a strain on the right heart and a right-sided cardiac dilatation is apparent. These are the chief characteristics of stage 1.

2. Even during stage 1 the injury to the alveolar membranes and the increased pressure have initiated the transfusion of fluid from the blood into the tissue spaces and later into the air passages of the lungs. The rapid development of this edema is the chief characteristic of stage 2. It results in hemoglobin concentration, reduction in blood volume and decrease in heart size, all three of which proceed to extreme degrees. Death ultimately results from decreased oxygenation of the pulmonary blood and from oxygen starvation of the tissues due to decreased blood volume, the latter, as Underhill states, being probably the more important.

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TO WHAT EXTENT ARE THE PHYSIOLOGICAL EFFECTS OF CARBON DIOXIDE DUE TO HYDROGEN IONS?

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INTRODUCTION

Carbon dioxide in aqueous solutions—and in living organisms there is always water to form such solutions—behaves as a weak acid, dissociating in the following manner:

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^{\cdot} + HCO_3' \quad \downarrow\uparrow \quad H^{\cdot} + CO_3''$$

It is a matter of some importance to determine to what extent its well-known physiological properties are due to the presence of hydrogen ions in its solutions, i.e., how far it behaves as an ordinary acid—and to what extent they may be of a more specific nature, due possibly to some peculiarity of the undissociated CO_2 or H_2CO_3 molecule.

This question is of especial interest in connection with the problem of the regulation of the respiratory movements of the higher vertebrates. The delicacy of the response of the mammalian respiratory center to changes in the CO_2 tension of the alveolar air and the blood was strikingly shown by the work of Haldane and Priestley (1). Several years later Winterstein (2) suggested that it is not to the CO_2, as such, but rather to an excess of hydrogen ions in the blood, that the response occurs. This conclusion was strongly supported by the work of Hasselbalch and Lundsgaard (3), (4) who showed that while the CO_2 content of the blood fluctuates considerably with different diets, etc., its hydrogen ion concentration is maintained practically constant; and this view of the rôle of hydrogen ions has been very generally accepted by physiologists, including Haldane (5). However, there have not been wanting supporters of the view that CO_2 may have a specific effect which is not due to hydrogen ions in its solutions. Among these may be mentioned Lacqueur and Verzár (6), Hooker (7) and Scott (8). The evi-

dence recently presented by the last two workers is of especial interest. Hooker and his collaborators found that for the same hydrogen ion concentration of the blood, the effect on the respiratory center of the dog is much greater with a high CO_2 tension than with a low one. They are inclined to attribute this result to some specific peculiarity of the CO_2 molecule, either in directly stimulating the respiratory center or in increasing its irritability to hydrogen ions. Scott, by a somewhat different method, found that in the decerebrate cat increased respiration may be brought about at a hydrogen ion concentration *lower* than that of normal blood, provided that sufficient quantities of CO_2 are present. He considers that probably both hydrogen ions and undissociated CO_2 molecules may serve as respiratory hormones.

In view of the general importance of the question of the nature of the effects of CO_2 on living protoplasm—of which its effects on the respiratory center are a particular case—it is desirable that further work should be done on material which, unlike the mammalian respiratory center, is open to direct observation. The experiments here described have been performed with this end in view on several varieties of material, including tadpoles, various protozoa, and the taste receptors of the human tongue. They may conveniently be described in the order mentioned.

EXPERIMENTS ON TADPOLES

Carbon dioxide in high concentrations is extremely toxic to young tadpoles of the toad (Bufo americanus). When placed in a quantity of distilled water saturated with CO_2 at atmospheric pressure, these animals show movements for a few seconds, and then settle to the bottom of the vessel, where they remain motionless for an indefinite period. If removed to fresh water within a minute or two, they usually recover; if the exposure lasts from three to five minutes or more at room temperature, death is the result. That the effects produced are not due merely to the absence of oxygen is shown by such an experiment as the following. A quantity of water was boiled and then placed in a test-tube in which it was cooled by passing through it a stream of hydrogen which had first been conducted through an alkaline pyrogallol solution. After the current of hydrogen had flowed for ten minutes, tadpoles were quickly introduced, the tube was restoppered and the current of hydrogen again allowed to flow. The amount of oxygen available for the animals under these conditions was practically negligible; nevertheless, movements continued for over ten minutes (as compared with a few seconds

for the CO_2 saturated water) and when the animals were removed at the end of fifteen minutes, all soon regained their normal activities and showed complete recovery.

To test the view that the loss of movement and death of the tadpoles when subjected to CO_2 solutions are due to hydrogen ions in the medium, solutions of hydrochloric, oxalic, formic, salicylic, acetic, butyric and carbonic acids (the last a saturated solution of CO_2 in distilled water) were made up to approximately the same pH (ca. 3.8–3.9) by means of the indicator, tetrabromphenolsulphonephthalein. Two tadpoles were placed in about 20 cc. of each solution in separate test tubes and the solutions were changed twice to insure the action of the full strength of the acids. The results of the experiment were that while the animals in the carbonic acid showed cessation of all movements in five seconds, those in all of the other acids were active at the end of thirty minutes. Those in butyric acid died within an hour, while the remainder lived for several hours. It appears, therefore, that the hydrogen ion concentration of a CO_2 solution is not the most important factor in determining its toxicity for tadpoles, since the same concentration when produced by other acids is incomparably less effective.

The lack of dependence of the effects of CO_2 on the hydrogen ion concentration of its solutions is further brought out by the following experiment. An M/4 solution of $NaHCO_3$ was saturated with CO_2 and was found by the indicator method to have a pH of approximately 6.9, i.e., it was almost neutral, the acidity being very much less than that of distilled water in which the tadpoles live for days. Nevertheless, loss of movement and death occurred in this solution in practically the same time as in the CO_2 saturated distilled water. A control experiment with the $NaHCO_3$ alone showed that it had produced no cessation of movement or visible injury in two hours. Incidentally, this last fact disposes of the possibility that H_2CO_3 might act chiefly through its anion, HCO_3', since this ion is present in abundance in bicarbonate solutions, which are seen to be practically without effect. The conclusion must therefore be drawn that for certain concentrations of CO_2, characteristic effects will be produced not merely at a pH which in the case of other acids is only very slightly effective, but even when the reaction of the medium is practically neutral. So far as toad tadpoles are concerned, CO_2 appears to be a substance of very different properties from ordinary acids. A discussion of the possible reasons for this difference may be reserved until after a description of the experiments made upon protozoa.

EXPERIMENTS ON PROTOZOA

Of over a dozen species of ciliate and flagellate protozoa studied by the author (9), all are killed when exposed to a current of CO_2 in an Engelmann gas chamber, the time required varying greatly with different forms. *Coleps*, for example, may be killed in two or three minutes, *Paramecium bursaria* usually in ten or fifteen, while under the same conditions *Paramecium caudatum* may survive an hour or more and *Colpidium* several hours. These relatively great differences in the resistance of the different forms furnish a means of further testing the possible mode of action of CO_2. If the effects of this substance are really due to the hydrogen ions in its solutions, then approximately the same order of resistance ought to be obtained with solutions of other acids, especially when the hydrogen ion concentrations are nearly the same.

Such, however, proves not to be the case. Forms which have a high resistance to CO_2 may have a low one to other acids, and vice versa. For example, the author (9) has shown that *Euplotes patella* is considerably more sensitive to CO_2 than *Paramecium caudatum*. Under comparable conditions, where the latter survives for an hour or more, the former may succumb in perhaps thirty minutes; i.e., its resistance is only approximately half as great. In the case of over a dozen other acids, however, recently studied by Miss Collett (10), the exact reverse is true. "At whatever concentration they are tested, no matter what the acid, Paramecium is much less resistant than Euplotes." The author can confirm from his own personal observations the high resistance of *Euplotes*, as compared with *Paramecium*. to mineral and to a number of organic acids. Only in the case of carbonic acid does this relation fail to hold, indicating the possession by this substance of certain unique properties not shared by the other acids.

Another example is furnished by the three common species of *Paramecium: P. caudatum, P. aurelia and P. bursaria.* To CO_2, the order of resistance, under comparable conditions is:

$$P.\ caudatum > P.\ aurelia > P.\ bursaria$$

In one experiment where all three forms were found in the same culture and had, therefore, for several weeks been under the same conditions, the average times of death when exposed to CO_2 were: *P. caudatum*, thirty-two minutes; *P. aurelia*, eighteen minutes; *P. bursaria*, six minutes. To N/10,000 HCl, the corresponding times in minutes were:

nine, five and twelve minutes respectively. In general, it has been found that for mineral acids the order of resistance is:

P. bursaria > *P. caudatum* > *P. aurelia*

That is to say, the form that is the most resistant to the mineral acids, which almost certainly act chiefly through their hydrogen ions, is *least* resistant to CO_2.

Still another example is furnished by *Coleps* which, while by far the most susceptible to CO_2 of the forms studied, is by no means unusual with respect to mineral acids, having approximately the same order of resistance as *Paramecium caudatum*. A number of other similar cases could be mentioned. Indeed, the first half-dozen forms compared showed almost an exact inverse relation between their susceptibility to CO_2 and to H_2SO_4. This probably was merely a coincidence, since other forms studied later did not show the same relation, but the fact comes out clearly that while different forms tend to agree in the order of their resistance to other acids, their resistance to CO_2 is apparently an unrelated quantity. It would appear, therefore, that as a toxic agent to the protozoa studied, carbonic acid is in a class by itself.

Further evidence on this point is furnished by the visible effects of CO_2 on certain other protozoa. A particularly striking case is that of *Peranema*, a common flagellate with a single flagellum and a body capable of being drawn into a great variety of shapes by the contractile myonemes with which it is provided. The author has previously pointed out (9) that the effect of CO_2 on *Peranema* is to cause an almost instantaneous paralysis of the myonemes, while leaving the flagellum free to beat for perhaps three-quarters of an hour. If this rather striking effect is due to hydrogen ions in the CO_2 solution, it should be possible to imitate it with acids like HCl or H_2SO_4 of the proper concentration. Attempts to do so, however, have shown that the effects of these acids are not only not similar, but are in some respects the reverse of those of CO_2. When exposed to them, at least if the concentration be sufficiently high, the flagellum usually stops beating, but the myonemes, instead of being paralyzed, are powerfully stimulated and the animals may make vigorous contortions for an hour or more. The failure to paralyze the myonemes, but the tendency rather to stimulate them was observed at all strengths of the acids employed from N/100 to less than N/10,000. At the last mentioned concentration it was possible for the flagellum to beat, but not normally.

Very similar effects were obtained with *Euglena*, a related green form, and with the ciliate *Vorticella*. In the latter, the contractile filament of the stalk is quickly paralyzed by CO_2, while the membranelles continue to beat for a long time, whereas in various strengths of HCl and H_2SO_4 the stalk is unusually active, almost up to the time of death, while the bell is for the most part closed and the activity of the membranelles clearly depressed. It appears therefore that while contractile structures like the myonemes of *Peranema* and *Euglena* and the contractile filament of *Vorticella* are quickly paralyzed by CO_2 long before other portions of the cell are inactivated, the effect of mineral acids is to stimulate them to abnormal activity almost up to the time of death of the animal. The general effects of carbonic acid, therefore, are not only quantitatively, but qualitatively different from those of other typical acids.

THE TASTE OF CARBON DIOXIDE SOLUTIONS

The results so far described leave little room for doubt that carbonic acid is different in its mode of action from the other acids studied. As to the reasons for this difference, there are several possible explanations. Leaving out of consideration, for the reasons already mentioned, the possibility that its peculiarities may be due to the anion HCO_3', there remain two plausible hypotheses: *a*, there may be some chemical peculiarity of the CO_2 or H_2CO_3 molecule as a whole which is responsible for its apparently specific action; or *b*, carbonic acid, once inside a cell, may behave essentially as other acids, but may differ from them in its much greater powers of penetrating not only cell membranes, but all other cell structures as well. To decide between these two hypotheses is not as simple a matter as it might appear to be. One possible method of testing the truth of the second one would be by the use of cells containing a natural indicator sensitive to carbonic acid. Unfortunately, the author has not as yet been able to secure material suitable for the employment of this method, though he hopes soon to be able to obtain it. Another method would be to place cells or tissues in a mixture of bicarbonate and carbonic acid and by means of indicators, or otherwise, to test the penetration of the latter, by noting any rise in the alkalinity of the mixture. There are certain practical difficulties, some obvious and some not so apparent, that have to the present time prevented the successful employment of this procedure. A third method, however, of extreme simplicity, has yielded definite results. It involves the use of certain taste receptors of the human tongue.

The connection between the sour taste of acids and the presence of hydrogen ions in their solutions has long been known. Over twenty years ago Richards (11) showed that surprisingly accurate titrations could be made by the sense of taste. In general, the greater the degree of dissociation of an acid, the more pronounced the sour taste. However, the concentration of hydrogen ions is not the only factor; acetic acid, for example, has a more powerful effect, as compared with mineral acids, than its hydrogen ion concentration would lead one to expect. The question of penetration probably plays an important part in producing the observed effects. Crozier (12) points out that the order of effectiveness, in giving a sour taste, of the series of acids studied by Becker and Herzog (13) is almost identical with that which he obtained in studying the order of penetration of acids into the pigmented cells of *Chromodoris zebra* (14). It is probable, therefore, that the two important factors in determining the sourness of a solution of an acid are *a*, the actual concentration of hydrogen ions in the solution, and *b*, the penetrating powers of the acid itself.

From these considerations it is apparent that the taste of CO_2 solutions might be utilized in throwing light on its powers of penetrating cells and on certain of its other physiological peculiarities. The first experiments made with this end in view rather favored the theory that the more striking effects of CO_2 are of a specific nature, and are not primarily due to hydrogen ions. The taste of a saturated solution of CO_2, as is well known, is not markedly sour; the most prominent effect in the mouth is the production of a stinging or a tingling sensation, which is entirely different from the sour taste of weak solutions of other acids. Furthermore, when such a saturated solution is diluted with distilled water to ten times its original volume, the sour taste disappears completely, though the tingling sensation is still perceptible. It might seem reasonable to conclude, therefore, that a carbonic acid solution has two effects: a weak one due to its hydrogen ions and a stronger one due to some other peculiarity of its molecules.

Such a conclusion, however, is unwarranted. If drops of a saturated solution of CO_2 be placed with a pipette on different parts of the tongue, or if a current of CO_2 from a generator be similarly directed, it appears that different parts of the tongue respond differently. On its tip the stinging sensation alone is perceived; along its sides and dorsally toward its base only the sour taste. The same relation holds for dilute solutions which, when taken into the mouth in the ordinary way, give no sour taste. Furthermore, an acid like HCl in concentrations of N/100 or

higher, when placed on the tip of the tongue, is not sour but gives the same stinging sensation that is obtained with CO_2. So far as its taste is concerned, therefore, carbonic acid does not appear to be qualitatively different from other acids; the differences are merely quantitative, and might reasonably be correlated with a more ready penetration of the taste receptors by this substance.

For purposes of comparison it might be of interest to state that according to Crozier (12) the hydrogen ion concentration of a solution of HCl which is just perceptibly sour (N/900) is 0.00119N and of the corresponding acetic acid solution (N/200) 0.00035N. Calculation shows that a solution of CO_2 one-tenth saturated at room temperature, in which a slightly sour taste can be detected on the proper part of the tongue, should have a hydrogen ion concentration of approximately 0.00003N; i.e., carbonic acid is about ten times as effective as acetic acid in giving a sour taste.

The hypothesis that CO_2 owes its unusual properties not to the hydrogen ion concentration of its solutions but rather to its great penetrating powers and to the hydrogen ion concentration produced within the cells, was tested by the following experiment. An M/2 $NaHCO_3$ solution was saturated with CO_2 and found by the indicator method to have a pH of approximately 7.4; i.e., it was on the alkaline side of neutrality. Nevertheless, such a solution on the sides and the posterior dorsal portion of the tongue gave a *distinct sour taste*, which was very apparent on alternately testing it and the original unsaturated bicarbonate solution, the latter having in comparison a decidedly flat taste.

The rather surprising result of an alkaline solution with a sour taste may plausibly be explained on the assumption that the CO_2 or H_2CO_3 readily penetrate the taste cells while the $NaHCO_3$ does not. In the external medium, the dissociation of the carbonic acid is strongly depressed by the bicarbonate; within the cells this restraint is no longer effective, and the hydrogen ions are free to produce their typical effect on the taste receptors. What is true in this case might very plausibly be supposed to be true also in the case of the respiratory center; i.e., the effective hydrogen ion concentration might be very different from that of the blood, provided that the acid concerned is the quickly penetrating carbonic acid. According to this theory, the results of Hooker and of Scott are readily intelligible and do not necessarily conflict with the orthodox view that hydrogen ions are the respiratory hormone. In one sense (i.e., in its unique powers of penetration)

CO_2 might be looked upon as having a specific effect, though acting within the cells like other acids by means of hydrogen ions.

The general mode of action of CO_2 may be pictured as follows. In the medium surrounding the cell are H; HCO'_3 and CO''_3 ions, and CO_2 and H_2CO_3 molecules. The ions, as such, do not penetrate to any appreciable extent, though the H ions, at least, acting on the cell surface, may have a stimulating effect. However, one or the other, or both, of the undissociated molecules may readily penetrate to all parts of the cell and there dissociate in the ordinary way giving hydrogen ions which produce effects not obtained at all with corresponding concentrations of other acids. A somewhat analogous case is perhaps furnished by "mustard gas." The usual explanation of the method of action of this substance is that in aqueous solutions it becomes hydrolyzed to HCl and dihydroxyethylsulphide; the HCl, at least in weak solutions, has very feeble powers of entering cells and an old solution is therefore relatively non-toxic. The unaltered molecules in a fresh solution, however, readily enter cells and when hydrolysis occurs within them, the HCl set free may have strongly toxic effects which may be imitated by the direct injection of this substance with a very fine pointed pipette (15). Whether or not this simple explanation is adequate to account for all of the peculiarities of mustard gas, it at least may be used to illustrate the possible mode of action of CO_2.

The similarities and differences between carbonic and other acids may be brought out very clearly by studying their visible effects on such an organism as *Paramecium*. A mineral acid, such as HCl or H_2SO_4 clearly acts from without inward, killing or injuring its way as it goes. One of the first effects produced by it is on the cilia, locomotion ceasing and their beat becoming slow and irregular. At a time when the beat of the cilia has almost ceased, the internal structures in the cell may be apparently unaffected. The contractile vacuoles may pulsate regularly, the protoplasm be clear and transparent and the nuclei possess their normal appearance. As the acid slowly enters the cell, the pulsations of the contractile vacuoles cease, swelling of the body occurs, the nuclei stand out sharply from the cytoplasm, and the latter becomes coagulated and opaque. With carbonic acid the results are very different, this substance acting in a sense from within outward. Animals exposed to it in the usual way in an Engelmann gas chamber quickly show visible internal signs of its effects. Swelling of the body begins almost immediately, and may continue until the buccal groove is almost obliterated; the nuclei soon stand out sharply, as when

treated with acetic acid; the contractile vacuoles become paralyzed; and even signs of coagulation of the protoplasm may appear—all before the cilia are sufficiently affected to bring about cessation of locomotion. Evidently the CO_2 enters immediately, the mineral acid much more slowly; though the final effects produced in the two cases are not dissimilar.

The differences between the effects of CO_2 and of ordinary acids on *Peranema*, *Vorticella*, etc., described above, may readily be explained by the greater penetrating powers of CO_2. It is not surprising, in the light of the facts already mentioned, that it is able to paralyze so quickly the myonemes of *Peranema* and *Euglena* and the contractile filament of the stalk of *Vorticella* (all of which are internal structures) while concentrations of mineral acids strong enough to stop the beat of the flagella and the membranelles are unable to do so, and manifest their presence for a long time merely by their stimulating action on the surface of the cell. The different order of resistance of different protozoa to CO_2 and to an acid such as HCl is also exactly what would be expected when it is remembered that the action of CO_2 is primarily internal and that of the other acid is primarily external and only secondarily internal. The results obtained with protozoa, therefore, do not conflict with the view here advanced as to the nature of the action of CO_2.

In conclusion it may be said that the general result of the experiments described in this paper is to indicate that while in certain respects CO_2 in aqueous solutions behaves as an ordinary acid, acting through its hydrogen ions, in other respects—especially in its remarkable powers of penetrating living cells—it may be said to have a specific action different from that of other acids. This theory makes it possible to reconcile what have been until now two apparently contradictory points of view as to the mode in which it produces its characteristic effects on the respiratory center of mammals, and on living protoplasm in general.

SUMMARY

1. A saturated solution of CO_2 is incomparably more toxic to toad tadpoles than are solutions of hydrochloric, oxalic, salicylic, formic, acetic or butyric acids of the same hydrogen ion concentration.

2. In the presence of sufficient bicarbonate to give practically a neutral reaction to the solution, the toxic properties of CO_2 to tadpoles are unchanged.

3. The order of resistance of various protozoa to CO_2 bears no relation to the order found in the case of other acids.

4. CO_2 very quickly paralyzes contractile structures in protozoan cells while causing little injury to flagella, etc. Under certain conditions mineral acids may produce apparently exactly the opposite effects.

5. The differences between carbonic and the other acids studied may probably be accounted for by a difference in their powers of penetrating living cells.

6. Due possibly to the ready penetration of the taste receptors by CO_2, and to its subsequent dissociation, an alkaline bicarbonate-carbonic acid mixture may have a distinct sour taste.

7. The hypothesis that similar conditions exist in the mammalian respiratory center would reconcile the two apparently conflicting views held at present as to the method in which CO_2 acts as a respiratory hormone.

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THE GASTRIC RESPONSE TO FOODS[1]

VII. The Response of the Normal Human Stomach to Vegetables Prepared in Different Ways

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The object of this investigation was to determine the response of the normal human stomach to the different members of the class of foods grouped together under the name of vegetables. These comprise a variety of leaves, stems, roots, bulbs, tubers and seeds, differing widely in chemical composition and valued in the diet for different reasons. An attempt was made to study the digestion of members of each of these classes. Wherever practicable comparisons of the raw and boiled vegetables were carried out and different methods of cooking were also employed.

Beaumont, in connection with his experiments on Alexis St. Martin, studied the digestion of certain vegetables in the stomach of his subject. Definite quantities of foods were not given in his test meals. These meals were also in most cases of a mixed character. Beaumont's results are given in table 1.

The results recorded by Beaumont agree, in hardly any respect, with the findings in our more carefully controlled experiments. Where Beaumont found a difference of an hour in favor of baked over boiled potatoes, we found no such advantage. Beaumont found boiled potatoes to remain in the stomach $3\frac{1}{2}$ hours. We found 100 grams of boiled potatoes to remain only $2\frac{1}{4}$ hours in the case of a man of slow-emptying type. Beets we found to leave the stomach rapidly (in from 1 to 2 hours). Beaumont found them to remain long ($3\frac{3}{4}$ hours). Parsnips we found to require distinctly longer than carrots or beets.

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Beaumont's results would indicate that the reverse was true. Beaumont gives 3½ hours for boiled turnips; we found 1½ hours. Beaumont found boiled cabbage to require 2 hours longer than raw. We found no such difference. Beaumont's figures would indicate that boiled rice and barley leave the stomach in a fraction of the time required for other vegetables. This finds no support in our experiments. This is not a criticism of Beaumont's valuable pioneer work but only of the uncritical use of his data.

Penzoldt (2) made a number of experiments on a single subject (Croce). In most cases he gave 150 gram portions (based on the raw weight). Samples were removed at half-hour periods. Hence no closer

TABLE 1

Digestion of vegetables in the human stomach (Beaumont)

FOOD	EVACUATION TIME
	hours and minutes
Potatoes, boiled	3:30
Potatoes, roasted	2:30
Potatoes, baked	2:30
Beets, boiled	3:45
Carrots, boiled	3:15
Parsnips, boiled	2:30
Turnips, boiled	3:30
Green corn and beans	3:45
Cabbage, raw, plain	2:30
Cabbage, raw, with vinegar	2:00
Cabbage, boiled	4:30
Rice, boiled	1:00
Barley, boiled	2:00

time comparisons were possible. The drinking of water by Penzoldt's subjects must also be considered, and the fact that most of his results are based on single experiments. The results obtained by this author are given in table 2.

The results of Penzoldt show a fair agreement with our own in the few cases where they are directly comparable.

The method of investigation used in our experiments was that previously described (3). More emphasis was laid on the microscopical examinations of samples which were made in nearly all cases. The subjects were normal medical students and laboratory workers who were at rest during the experimental period.

The results as regards gastric acidities developed and evacuation times are given in table 1. The distinction between subjects of rapid- and slow-emptying types is less in the case of most vegetables than in the case of meats. The classification of subjects is based upon the response to other foods as well as vegetables.

Comparative responses of the same individuals to different forms of vegetables are charted in figures 1 to 31. In these figures the prime or accent marks indicate curves of free acidity.

TABLE 2

Digestion of vegetables in the human stomach (Penzoldt)

FOOD	AMOUNT	EVACUATION TIME
	grams	*hours and minutes*
Potatoes, boiled	150	2:05
Potatoes, mashed	150	2:45
Potatoes, "Gemüse"	150	3:20
Carrots, boiled	150	3:20
Radishes, raw	150	3:05
Lentils, ground and acidified	150	4:05
Peas, boiled	200	4:15
Beans, string, boiled	150	4:20
Kohlrabi, boiled	150	3:05
Cauliflower, "Gemüse"	150	2:35
Cauliflower, boiled	150	2:25
Cauliflower, vinegar and oil	150	2:05
Asparagus, boiled	150	2:45
Asparagus, oil and vinegar	150	3:00
Spinach, boiled	150	3:30
Cucumbers as salad	150	3:15
Rice, boiled	100	3:35

Potatoes, whole boiled, baked, mashed and fried. Boiled whole white potatoes left the stomach rather quickly requiring $2\frac{1}{4}$ hours for a slow-emptying man (fig. 1). The same man required $1\frac{3}{4}$ hours for creamed potatoes. For baked potatoes, plain, he required $2\frac{1}{4}$ hours but baked potatoes with butter remained $3\frac{1}{4}$ hours. Another subject (fig. 11) required $1\frac{1}{2}$ hours for creamed potatoes and but 15 minutes longer for baked potatoes with butter. It appears therefore that boiled potatoes and creamed potatoes are handled in about the same time as the plain baked potatoes but that the addition of butter causes a slight delay. A potato salad required 2 hours as compared with $2\frac{1}{4}$

hours for mashed potatoes, on one subject (figs. 3 and 4) while in the case of a slow-emptying type of stomach 3 hours were found necessary for the mashed potatoes and $3\frac{1}{2}$ hours for the salad (fig. 5). Plain mashed potatoes were compared with mashed potatoes to which milk and butter had been added (figs. 3 and 5). In one case these additions delayed evacuation 15 minutes, in the other they hastened it 15 minutes. The influence of the milk and butter hence cannot have been marked.

Fried potatoes are frequently stated to be more difficult of digestion than boiled or mashed potatoes. We were, therefore, somewhat surprised to find that on the average fried potatoes left the stomach fully as rapidly as potatoes cooked in other ways (see figs. 4, 5, 6, 7 and 8). One subject (figs. 3 and 4) required $1\frac{1}{2}$ to $2\frac{1}{2}$ hours for fried potatoes as compared with 2 to $2\frac{1}{2}$ hours for mashed potatoes and potato salad. Another subject (fig. 5) required a little longer for French fried potatoes than for mashed potatoes but no longer than for potato salad. Subject McEl (figs. 6 and 7) showed an emptying time of $2\frac{1}{4}$ hours for plain or German fried potatoes as compared with $1\frac{3}{4}$ hours for boiled and 2 hours for baked sweet potatoes. Subject Mil (fig. 8) required $2\frac{1}{4}$ hours for either French fried or boiled potatoes.

The order of rapidity of digestion of different kinds of fried potatoes on one subject (figs. 3 and 4) was German fried, first, plain fried, second and French fried, third. Another subject showed exactly the same response to German fried and plain fried potatoes. It is possible that the French fried potatoes require a few minutes longer than the others but the difference cannot be marked.

Potato chips were also compared with French fried potatoes. Because of their high content of dry matter, especially fat, only 50 grams of the chips were fed. They required $2\frac{1}{2}$ hours to digest as compared with $2\frac{1}{4}$ hours for the fried potatoes (fig. 8). This would indicate that potato chips are handled rather readily by the stomach considering their fat content of about 40 per cent.

Sweet potatoes, dasheens and cassava roots. Boiled sweet potatoes remained in the stomach $2\frac{3}{4}$ hours as compared with $2\frac{1}{4}$ hours for boiled white potatoes (figs. 1 and 2). Baked sweet potatoes with butter required $2\frac{3}{4}$ hours as compared with $1\frac{3}{4}$ hours for baked white potatoes with butter (fig. 11). Plain fried sweet potatoes required, with the same subject, 2 hours. Sweet potatoes in general therefore appear to be retained in the stomach longer than white potatoes cooked in the same way.

Dasheens were cooked in several ways, namely, boiled, baked, riced, plain fried and French fried. The boiled dasheen required, for a subject of slow-emptying type (see fig. 5) $3\frac{1}{4}$ hours as compared with $2\frac{3}{4}$ to $3\frac{1}{2}$ hours for white potatoes prepared in different ways. In another case baked dasheens required $2\frac{3}{4}$ hours as compared with $2\frac{1}{4}$ hours for baked white potatoes and $2\frac{3}{4}$ hours for boiled sweet potatoes (fig. 2). A third subject (see figs. 3 and 4) showed an evacuation time of 3 hours for baked dasheen as compared with $1\frac{1}{2}$ to $2\frac{1}{2}$ hours for white potatoes prepared in different ways and $3\frac{1}{2}$ hours for baked sweet potatoes with butter. Riced dasheen took a shorter period of time, $2\frac{3}{4}$ hours, as compared with $1\frac{3}{4}$ to $2\frac{1}{4}$ hours for white potatoes and 2 hours for baked sweet potatoes (see figs. 6 and 7). French fried dasheens took distinctly longer to leave the stomach than white potatoes prepared in the same way (see fig. 8). Another subject of the rapid-emptying type showed very little difference in the emptying time of dasheens as compared with potatoes (see fig. 11). In general dasheens appear to require a longer period of gastric digestion than white potatoes but no longer than sweet potatoes.

Cassava root was tested in the boiled and baked forms. A subject of the rapid-emptying type showed an evacuation time for boiled cassava of $2\frac{1}{2}$ hours (see table 3). A subject of the slow-emptying type required 3 hours as compared with $2\frac{1}{4}$ hours for boiled white potatoes (figs. 1 and 2). Baked cassava with butter required $2\frac{3}{4}$ hours as compared with $1\frac{3}{4}$ to $2\frac{1}{4}$ hours for boiled or fried white potatoes (figs. 6 and 7). The cassava root thus requires a little longer to digest in the stomach than white potatoes similarly prepared.

Boiled oyster plant required about the same period of gastric digestion as white potatoes (see fig. 11).

Beets, carrots, parsnips, radishes and turnips. Beets whether plain boiled or pickled left the stomach rapidly, that is, in from 1 to $1\frac{1}{2}$ hours for subjects of the rapid type and 2 hours for a subject of the slower type (see figs. 9, 13, 24 and 10). Pickling reduced the time required for beets to leave the stomach (fig. 13). This may be due to the acidity of the pickled beets.

Carrots were fed raw and boiled. The raw carrots left rapidly, that is, in from $1\frac{1}{2}$ to 2 hours (figs. 14 and 15). Boiled carrots took the same subjects distinctly longer, that is, $2\frac{1}{4}$ to $2\frac{1}{2}$ hours. This may be due to the greater acid-combining power of the boiled carrots, as is indicated by the much higher acid curves obtained with the boiled vegetables. These results do not confirm the statement of Hutchison

that carrots are not easily disposed of by the stomach (4), though it is true the cellular masses disintegrate slowly.

Boiled parsnips were tested on two subjects. In each case these vegetables remained in the stomach 3 hours (figs. 14 and 24). Parsnips thus required distinctly longer than beets or carrots. This may be because parsnips are more irritating to the mucosa of the intestine.

Radishes were given in 50 gram portions to two subjects. In one case (figs. 15 and 16) the radishes left the stomach in $1\frac{1}{4}$ hours or much more rapidly than 100 grams of carrots or sugar corn, etc. The acidity developed was low in this case but moderate in the other subject (fig. 10). In the latter case the radishes left in $1\frac{1}{2}$ hours or more quickly than any vegetables except red beets.

An alcoholic extract of the radish peel furnishes a very sensitive indicator for acid and alkali titrations as pointed out by Sacher (3) whose results we were able in a general way to confirm. As the pink color of the acid solution changes to blue in an hydroxyl-ion concentration less than that required for phenolphthalein it did not interfere appreciably with our titrations for total acidity.

Boiled turnips left the stomach rapidly. In both cases (figs. 9 and 12) 100 grams of turnips left in $1\frac{1}{2}$ hours. This may be attributed to the low content of turnips in substances combining with acid.

Beans, peas, endives and lentils. Boston baked beans were tried out on two individuals (see figs. 17 and 23). As might be expected from their high protein content, these beans remained somewhat longer in the stomach than most other forms of vegetables and developed higher combined acidities. Thus where beans required $3\frac{1}{2}$ hours, cabbage raw or boiled required $1\frac{1}{2}$ hours (fig. 23). Baked lima beans were tested on one subject of the rapid-emptying type and while only 2 hours were required by this subject to digest the beans, a shorter period was required for nearly all of the other vegetables tested (see figs. 11 and 12).

String beans, on the other hand, left the stomach rapidly resembling the green vegetables in their stimulatory power upon the stomach (see figs. 8, 9 and 18). Any view that string beans normally remain a long time in the stomach is therefore shown to be without foundation.

Boiled peas left the stomach in $1\frac{3}{4}$ hours in each of the two cases where they were fed. In each case they were handled more rapidly than beans (figs. 11, 12 and 17).

But a single experiment was carried out on boiled lentils (see fig. 21). These required $2\frac{1}{2}$ hours to leave the stomach or a little longer than

TABLE 3

Response of the human stomach to vegetables

NUMBER	SUBJECT	FOOD AND PREPARATION	RAPID-EMPTYING TYPE				SLOW-EMPTYING TYPE			
			Evacuation time, hours and minutes		Highest total acidity		Evacuation time, hours and minutes		Highest total acidity	
				average		*average*		*average*		*average*
1	Ev	Potatoes, whole, boiled					2:15	2:15	113	113
2	Ev	Potatoes, creamed					1:45	1:45	107	107
3	Lei	Potatoes, creamed	1:30	1:30	105	105				
4	Ev	Potatoes, baked, plain					2:15	2:15	108	108
5	Lei	Potatoes, baked, and butter	1:45		88					
6	Mi	Potatoes, baked, and butter	2:15	2:00	91	90				
7	Ev	Potatoes, baked, and butter					3:15	3:15	118	118
8	Sok	Potatoes, salad	2:00	2:00	99	99				
9	Dur	Potatoes, salad					3:30	3:30	109	109
10	Sok	Potatoes, mashed, plain	2:15	2:15	97	97				
11	Dur	Potatoes, mashed, plain					3:00	3:00	120	120
12	Sok	Potatoes, mashed, with milk and butter	2:30	2:30	89	89				
13	Dur	Potatoes, mashed, with milk and butter					2:45	2:45	93	93
14	McEl	Potatoes, plain, fried					2:15	2:15	72	72
15	Sok	Potatoes, plain, fried	2:00	2:00	106	106				
16	Mi	Potatoes, French fried	2:15		41					
17	Sok	Potatoes, French fried	2:30	2:20	92	67				
18	Dur	Potatoes, French fried					3:45	3:45	122	122
19	McEl	Potatoes, German fried					2:15	2:15	96	96
20	Sok	Potatoes, German fried	1:30	1:30	98	98				
21	Mi	Potato chips	2:30	2:30	55	55				
22	Ev	Sweet potatoes, boiled					2:45	2:45	105	105
23	Lei	Sweet potatoes, baked, and butter	2:45	2:45	56	56				
24	Lei	Sweet potatoes, plain, fried	2:00	2:00	41	41				
25	Dur	Dasheen, boiled					3:15	3:15	124	124
26	Ev	Dasheen, baked					2:45	2:45	118	118

27	Sok	Dasheen, baked	3:15	3:15	104	104	2:45	2:45	104	104
28	McEl	Dasheen, riced	1:45	1:45	90	90				
29	Lei	Dasheen, plain, fried	3:15	3:15	52	52				
30	Mi	Dasheen, French fried	2:30	2:30	97	97				
31	Wat	Cassava, boiled					3:00	3:00	65	65
32	Ev	Cassava, boiled					2:45	2:45	105	105
33	McEl	Cassava, baked, and butter	2:00	2:00	71	71				
34	Lei	Oyster plant, boiled	1:30		56					
35	Get	Beets, red, canned, boiled	1:15		88					
36	Mat	Beets, red, canned, boiled	1:30	1:25	43	62				
37	Mi	Beets, red, canned, boiled					2:00	2:00	64	64
38	Ema	Beets, red, canned, boiled	1:00		28					
39	Get	Beets, pickled	1:00	1:00	62	45				
40	O'Br	Beets, pickled	1:15		23					
41	Kru	Carrots, raw	2:00	1:40	57	40				
42	Wat	Carrots, raw	2:15		85					
43	Kru	Carrots, boiled	2:30	2:25	82	83				
44	Wat	Carrots, boiled	3:00	3:00	103	103				
45	Dur	Parsnips, boiled					3:00	3:00	103	103
46	Ema	Parsnips, boiled	1:15		40					
47	Wat	Radishes, raw, 50 grams	1:30	1:25	88	64				
48	Mat	Radishes, raw, 50 grams	1:30		67					
49	Lei	Turnips, boiled	1:30	1:30	61	64				
50	Mi	Turnips, boiled	2:15	2:15	90	90				
51	O'Br	Beans, baked					3:30	3:30	86	86
52	Ema	Beans, baked	2:00	2:00	102	102				
53	Lei	Beans, lima, canned	1:15		59					
54	Fla	Beans, string, cooked	2:00	1:40	89	74				
55	Mi	Beans, string, cooked	1:45		69					
56	Ow	Endives, raw	2:00	1:55	72	70				
57	Mur	Endives, raw	2:30	2:30	72	72				
58	Kar	Lentils, boiled	1:45		107					
59	Lei	Peas, canned, boiled	1:45	1:45	115	115				
60	O'Br	Peas, canned, boiled								

TABLE 3—*Concluded*

NUMBER	SUBJECT	FOOD AND PREPARATION	RAPID-EMPTYING TYPE				SLOW-EMPTYING TYPE			
			Evacuation time, hours and minutes		Highest total acidity		Evacuation time, hours and minutes		Highest total acidity	
				average		*average*		*average*		*average*
61	Wha	Asparagus, canned	1:30	1:30	36	36				
62	Fla	Cabbage, raw	1:15	1:15	79	79				
63	Em	Cabbage, raw					1:15		63	
64	Daly	Cabbage, raw					2:00		98	
65	Rud	Cabbage, raw					1:45	1:40	90	84
66	Rud	Cabbage, raw (250 grams)					3:00	3:00	119	119
67	Fla	Cabbage, boiled	1:15	1:15	62	62				
68	Ema	Cabbage, boiled					1:30	1:30	61	61
69	Fla	Cold slaw	2:15	2:15	63	63				
70	Ema	Sauerkraut and vinegar					2:00	2:00	118	118
71	Wha	Cauliflower, boiled	1:45	1:45	92	92				
72	Ema	Cauliflower, boiled					3:30	3:30	79	79
73	Grab	Celery, raw					3:00	3:00	91	91
74	Mat	Celery, raw	3:00	3:00	79	79				
75	Mat	Celery, creamed	1:45	1:45	96	96				
76	Grab	Celery, creamed					2:45	2:45	84	84
77	Fla	Lettuce, raw, plain	1:15	1:15	35	35				
78	Ema	Lettuce, raw, plain					2:15	2:15	72	72
79	Fla	Lettuce, sugar and vinegar	2:00	2:00	41	41				
80	Fla	Lettuce, olive oil and vinegar	1:45	1:45	56	56				
81	Ema	Lettuce, olive oil and vinegar					3:00	3:00	48	48
82	Ev	Spinach, boiled					2:45	2:45	103	103
83	McEl	Spinach, boiled					3:15	3:15		
84	Sok	Spinach, boiled	2:00	2:00	92	92				
85	O'Br	Onions, stewed	1:45		82					
86	Wha	Onions, stewed	2:45	2:15	60	71				

87	Gold	Onions, fried (25 grams)	1:30	1:30	50	50	2:30	2:30	78	78
88	Wil	Onions, fried (25 grams)								
89	Lei	Chayotes, creamed	1:15		70					
90	Kru	Chayotes, creamed	1:30		22					
91	Mi	Chayotes, creamed	2:00		51					
92	Wat	Chayotes, creamed	2:00	1:40	59	50				
93	Get	Cucumbers, raw, plain	1:45	1:45	71	71	3:15	3:15	58	58
94	Kiel	Cucumbers, raw, plain								
95	Get	Cucumbers, raw, salted	1:30	1:30	43	43	3:00	3:00	39	39
96	Kiel	Cucumbers, raw, salted								
97	Kru	Squash, boiled	2:15		97					
98	Wat	Squash, boiled	2:30	2:25	97	97				
99	Kru	Tomatoes, raw	1:00		50					
100	Wat	Tomatoes, raw	1:15	1:10	49	40				
101	Mal	Tomatoes, raw					2:00		84	
102	Kiel	Tomatoes, raw					1:45	1:55	54	69
103	Mal	Tomatoes, stewed, canned					2:30		25	35
104	Kiel	Tomatoes, stewed, canned					3:30	3:00	50	42
105	Son	Mushrooms, sauté (50 grams)	1:45	1:45	100	100	3:00	3:00	93	93
106	Far	Mushrooms, sauté (50 grams)								
107	Mur	Mushrooms, sauté (100 grams)	2:15		95					
108	Isr	Mushrooms, sauté (100 grams)	2:15	2:15	89	92				
109	Mir	Barley, boiled	1:30	1:30	53	53	3:00	3:00	60	60
110	Col	Barley, boiled								
111	Kru	Corn, sugar, canned	2:30		79					
112	Wat	Corn, sugar, canned	1:45	2:10	91	85	2:30	2:30	46	46
113	Kiel	Corn, sugar, canned								
114	Kara	Hominy, boiled	2:00	2:00	74	74	2:30		51	
115	McG	Rice, boiled, unpolished					3:15		80	
116	Col	Rice, boiled, unpolished					2:30	2:45	8	56
117	Lea	Rice, boiled, unpolished					1:45		72	
118	Ev	Rice, boiled, polished					1:00	2:50	39	55
119	Lea	Rice, boiled, polished					1:50	1:30	42	42
120	McG	Agar-agar								

* Acidities are expressed as cubic centimeters of N/10 alkali required to neutralize 100 cc. of sample.

peas. Raw endives required $1\frac{3}{4}$ to 2 hours in the cases of two individuals of the rapid-emptying type. They thus resembled peas as far as evacuation time was concerned (see figs. 19 and 20).

Cabbage, lettuce, asparagus and cauliflower. Asparagus (a canned variety) left the stomach rapidly, requiring but $1\frac{1}{2}$ hours (see fig. 22). Boiled cauliflower required for the same subject a few minutes longer. In the case of a subject of a different type (see fig. 24) the cauliflower took as long as baked beans and much longer than cabbage.

The digestion of cabbage was studied and comparisons made of the gastric response to the raw and boiled forms of this vegetable. Even subjects who showed slow evacuation on most other types of foods had no trouble in evacuating cabbage. Thus a subject who required $3\frac{1}{2}$ hours for either baked beans or cauliflower required only $1\frac{1}{4}$ hours for raw cabbage (see fig 23). Another subject who retained stewed veal $3\frac{1}{2}$ hours, retained cabbage but $1\frac{1}{4}$ hours (see fig. 18). Boiled cabbage required in one case the same period of digestion as the raw, in another case, however, 15 minutes longer. Boiling does not seem to increase the readiness with which foods of this character are handled by the stomach. That cooking decreases the nutritive value through loss of protein, carbohydrate, salts and antiscorbutics is well known.

Cold slaw required an hour longer to leave the stomach than plain raw cabbage (fig. 18). This can only be due to the added ingredients. Sauerkraut with vinegar required for one subject a gastric digestion period of 2 hours as compared with $1\frac{1}{4}$ hours for plain raw cabbage.

Lettuce was given to two subjects, comparisons being made of plain lettuce with lettuce, sugar and vinegar, and with lettuce, olive oil and vinegar. A subject of the rapid-emptying type (fig. 18) showed a gastric digestion time of $1\frac{1}{4}$ hours for plain lettuce as compared with 2 hours for the sugar-vinegar preparation and $1\frac{3}{4}$ hours when the leaves were treated with olive oil and vinegar. A subject of a different type (fig. 23) showed an evacuation time of $2\frac{1}{4}$ hours for the plain lettuce and 3 hours when oil and vinegar were added. The lettuce alone therefore threw a lesser burden upon the stomach and lettuce belongs to the most easily evacuated class of foods in spite of its great bulk as compared with equal weights of many such foods. This is related to the fact that such lettuce leaves the stomach almost unchanged and free acidities are quickly developed in the stomach after eating lettuce due to the low acid-combining power of the leaves. The response of the stomach to lettuce is, as indicated by the charts, very similar to that of cabbage in the same individuals.

Celery and spinach and onions. Raw and creamed celery were compared on two different subjects (see figs. 10 and 25). In both cases the creamed celery left the stomach sooner than the raw and in one case the difference was marked. This in spite of the fact that while 100 grams of the creamed product were fed in each case only 84 and 69 grams respectively of the raw stalk were ingested. In considering the rather slow evacuation of raw celery its pronounced flavor, high cellulose content, and the fact that large amounts of it are not ordinarily eaten at one time, must be borne in mind.

Boiled spinach was given to three subjects (see figs. 1 and 6). It remained in the stomach from 2 to $3\frac{1}{4}$ hours, which was distinctly longer than for most other foods of the vegetable class.

Stewed and fried onions were fed to four subjects. Only 25 grams of the fried onions were fed. The stewed onions remained in the stomach longer than other vegetables low in protein, such as cauliflower or asparagus but not longer than such high protein vegetables as beans and peas (see figs. 17 and 22). The fried onions required from $1\frac{1}{2}$ to $2\frac{1}{2}$ hours to leave the stomach. It seems clear that while the irritating character of the onion delays its passage somewhat this delay is not notable where moderate amounts only of onions are taken.

Chayotes, cucumbers, squash and tomatoes. Chayotes were creamed and in this form tested out on four individuals of the rapid-emptying type. Evacuation times varied from $1\frac{1}{4}$ to 2 hours. The acid responses were relatively low (see figs. 8, 9, 12, 14 and 16). The chayote belongs, therefore, in a class with those vegetables which leave the stomach most rapidly.

Cucumbers were given to two subjects each receiving first the raw sliced fruit and then on a later day the same amount of sliced cucumber which had been salted over night, the view being prevalent that such treatment renders this vegetable more digestible. The salted cucumbers did in fact leave the stomachs of these men a quarter of an hour sooner than the unsalted (figs. 13 and 26). It does not seem likely that this slight difference in evacuation time can be of great significance. The cucumbers left the stomach somewhat less rapidly than boiled corn or beets but much more readily than a meat. These vegetables do not therefore appear to impose any special burden upon the stomach. No ill effects were noted in any case.

In Korea "cucumbers are the most favored vegetables, and at one meal one gets them prepared in three or four ways—cucumber soup, salted cucumbers, fresh sliced cucumber and cucumber water. From

a baby who is hardly able to walk, up to the old, gray-haired men, everybody eats cucumbers and preferably unpeeled" (6).

Boiled squash remained in the stomachs of two subjects of the rapid-emptying type for 2¼ and 2½ hours respectively (figs. 14, 15 and 16). This was about the same time as required for boiled carrots or sugar corn and a little longer than for raw carrots or tomatoes.

Tomatoes were fed to four men. Two of these subjects were fed raw tomatoes and then a few days later an equal quantity of the boiled fruit. Again the boiling of a vegetable was found to retard evacuation in this case by ½ to ¾ hour (figs. 27 and 28). Raw tomatoes, it will be noted, left the stomach rapidly even with subjects of the slow-emptying type, this rapidity of evacuation being quite striking as compared with most of the foods tested.

Mushrooms. Mushrooms were fed to four subjects in amounts of 50 to 100 grams. In one case these mushrooms required 15 minutes longer to leave the stomach than raw endives which in turn gave a response similar to that of peas. In the other cases no direct comparisons were made with other vegetables as the principal object was to test the influence of mushrooms on the digestibility of meats which results will be published in another connection. It is clear, however, that subjects of the rapid-emptying type may require no longer than 2¼ hours to evacuate as large amounts of mushrooms as 100 grams and hence these fungi cannot be said to throw any unusual burdens upon the stomach.

Rice, polished and unpolished, corn and barley. This group of foods consists, of course, of cereal foods but inasmuch as they are frequently used as substitutes for certain of the common vegetables some comparative tests may be of interest in this connection.

Boiled barley was tried out in only two cases. A subject of the rapid-emptying type evacuated this food in 1½ hours while a subject of the slow-emptying type required 3 hours (fig. 30) or about the same time as for unpolished rice.

Canned sugar corn was fed to three subjects. The best comparison is given by figures 15 and 16, showing the corn to leave the stomach in about the same time as raw carrots or tomatoes but more rapidly than boiled carrots or boiled squash.

Boiled hominy required 2 hours in the case of a subject of the rapid-emptying type and thus would appear to leave the stomach almost as rapidly as sugar corn.

Boiled polished and unpolished rice was fed to four subjects all of the slow-emptying type. One comparison of the two on the same subject would indicate that the unpolished rice remains in the stomach for a distinctly shorter time or $2\frac{1}{2}$ hours as compared with 4 hours for the polished rice. In another case, however (fig. 2), the polished rice was evacuated in a relatively short time. One man emptied unpolished rice in about the same time as boiled barley (fig. 30) while figure 31 illustrates the response of a subject who required but $2\frac{1}{2}$ hours for unpolished rice. It appears that unpolished rice is readily handled by the average stomach and usually passes on into the intestine sooner than polished rice similarly cooked.

Agar-agar. Inasmuch as many of the vegetables tested contained cellulose as a chief constituent, and in spite of the slight action of the gastric juice upon such cellulose, they left the stomach rapidly, it was thought of interest to determine the response of the stomach to nearly pure cellulose or hemicellulose. For this purpose agar-agar soaked in water was chosen. A comparison was made of the agar-agar with boiled unpolished rice (fig. 31). While the rice required $2\frac{1}{4}$ hours and developed an acidity of 50, the agar-agar required only $1\frac{1}{2}$ hours showing an acid development of 60. This rapid evacuation of hemicellulose is believed to throw considerable light on the problem of the reasons for the typical response of the stomach to certain classes of vegetables.

Microscopical. The samples of gastric contents withdrawn for fractional analysis were also in most cases examined microscopically with a view to determining the degree of disintegration, the extent of starch digestion and other changes.

Most of the vegetables which were fed in the raw condition, although they left the stomach rapidly, showed slow disintegration of the cells and cellular masses. This resistance to gastric digestion was noted for raw carrots, celery, tomatoes, lettuce, cabbage and cucumbers. Boiled carrots and cabbage showed much more complete disintegration and left the stomach later. Creamed celery and chayotes were also rather slowly broken up. In most cases boiled vegetables exhibited a rapid cellular disintegration. This was noted, for example, with boiled white and sweet potatoes, turnips, squash, peas and dasheen. Somewhat less rapidly broken up were fried potatoes, spinach, string beans, oyster plant, parsnips, baked beans, asparagus, beets, sugar corn and cauliflower. Potato chips were resistant due to their fat content. With stewed onions the larger cells were soon broken but the small cells were more resistant.

The starchy vegetables such as the potatoes were found to be considerably affected by salivary digestion. The starch-iodine reaction frequently became very slight early in gastric digestion, the red dextrin color only being obtained. In other cases the starch reaction decreased markedly by the end of one hour or toward the end of digestion. Baked beans and sugar corn showed considerable starch digestion although some cells giving the blue iodine reaction could nearly always be found.

The formol titration. The formal titration for amino acid groups was carried out in every case. In general it may be said that these values were either low and fairly constant throughout digestion (as might be expected in the case of foods of low protein content) or the values began high and gradually decreased to a low value. The latter we believe represent in the main cases where the residuum at starting was high in digestion products which became gradually diluted although in some cases it was due to a high initial concentration of amino substances in the foods themselves. Nothing like the marked progressive development of amino acid nitrogen occurring in the case of meats, etc., was noted for vegetables.

SUMMARY AND CONCLUSIONS

A study was made of the response of the normal human stomach to thirty different kinds of vegetables prepared in different ways. One hundred and twenty-four experiments were made on twenty-five normal men. The evacuation times and acid responses of the stomach were determined and physical and chemical changes in the ingested food noted. Subjects were classified as belonging to the slow- and rapid-emptying types.

The average evacuation time for all subjects of the rapid-emptying type was 2 hours and for the slow-emptying type 2½ hours. The averages of the high points of the total acid curves were 70 and 77 respectively.

The response of the stomach to potatoes prepared in the following different ways was determined: whole boiled, creamed, mashed (with and without milk and butter), baked (with and without butter), potato salad, French, German and plain fried potatoes, and potato chips. All of these left the stomach in moderate time or 1½ to 2½ hours for rapid type individuals and 2 to 3½ hours for the slow type. Baked potatoes with butter required more than the average time but plain baked potatoes about the same time as boiled potatoes. The addition of

milk and butter to mashed potatoes had little effect nor did creamed potatoes or potato salad differ much from plain boiled potatoes as regards gastric response. Fried potatoes left the stomach as rapidly as potatoes prepared in other ways. French fried potatoes a few minutes longer than German or plain fried potatoes. Potato chips were handled very rapidly by the stomach considering their high solid and fat content.

Sweet potatoes whether boiled, baked or fried remained longer in the stomach than white potatoes cooked in the same ways. Dasheens, whether boiled, baked or fried required a longer period of gastric digestion than white potatoes but no longer than sweet potatoes. Cassava roots were fed boiled and baked and found to require a little longer than white potatoes similarly prepared. Boiled oyster plant required about the same period of gastric digestion as white potatoes.

Boiled red beets left the stomach rapidly, i.e., in from 1 to 2 hours. After being pickled in vinegar they left still more rapidly, the vinegar evidently hastening the evacuation. Raw carrots also left the stomach rapidly (in from $1\frac{1}{2}$ to 2 hours). Boiled carrots took distinctly longer ($2\frac{1}{4}$ to $2\frac{1}{2}$ hours). Boiled parsnips required distinctly longer than beets or carrots. Boiled turnips left the stomach in $1\frac{1}{2}$ hours. Radishes in amounts of 50 grams left the stomach very rapidly (in from $1\frac{1}{4}$ to $1\frac{1}{2}$ hours).

Baked beans, as might be expected from their high protein content, remained somewhat longer in the stomach than most other kinds of vegetables and developed higher combined acidities. String beans, on the other hand, left the stomach rapidly resembling more closely the green vegetables in stimulatory power upon the stomach. Boiled peas required $1\frac{3}{4}$ hours in each of two cases or less time than baked beans. Boiled lentils appeared to require a little longer than peas while raw endives resembled peas as far as evacuation times were concerned.

Asparagus left the stomach rapidly. Boiled cauliflower required only a few minutes longer in one case, but in a subject of the slow type showed delayed evacuation.

Cabbage whether raw or cooked left the stomach rapidly even in subjects of the slow-emptying type. Boiled cabbage was not handled more readily than the raw. Cold slaw and sauerkraut required distinctly longer to leave the stomach than plain cabbage.

Lettuce in spite of its bulky character was found to be one of the most easily evacuated of foods, the response to this food resembling

that to cabbage. The addition of sugar and vinegar or oil and vinegar delayed evacuation.

Celery in the raw state is evacuated more slowly than cabbage or lettuce but creamed celery caused less difficulty. Spinach remained in the stomach distinctly longer than most vegetables.

Onions stewed and fried showed slower evacuation than other vegetables low in protein, but not longer than high protein vegetables, when fed in moderate amounts.

Creamed chayotes left the stomach rapidly (in from $1\frac{1}{4}$ to 2 hours), cucumbers did not impose any special burden on the stomach, whether salted or unsalted. The unsalted required but a few minutes longer than the salted cucumbers.

Boiled squash required $2\frac{1}{4}$ to $2\frac{1}{2}$ hours or about the same time as boiled carrots or sugar corn.

Raw tomatoes left the stomach very rapidly in all cases. Boiled tomatoes required a longer time.

Mushrooms require only a moderate period of gastric digestion—$2\frac{1}{4}$ hours with a subject of the rapid type.

Stewed corn left the stomach almost as rapidly as raw carrots or tomatoes and boiled hominy required but little longer.

Boiled unpolished rice and boiled barley were readily handled by the stomach and usually passed into the intestine sooner than boiled polished rice.

Raw agar-agar left the stomach in $1\frac{1}{2}$ hours as compared with $2\frac{1}{2}$ hours for boiled unpolished rice. Hemicelluloses appear therefore to leave the stomach very rapidly and show a certain analogy with vegetables such as cabbage and lettuce.

In general raw vegetables low in protein, as carrots, celery, tomatoes, cabbage, lettuce and cucumbers, leave the stomach rapidly, develop moderately high free acidities but little combined acidity and leave the stomach without great change. Boiled vegetables show much more rapid and complete disintegration. Vegetables high in starch such as potatoes show very considerable starch digestion before leaving the stomach. In certain cases hardly any starch reaction could be obtained toward the end of digestion.

The authors desire to thank, for their coöperation, the many medical students who sacrificed time and convenience to serve as subjects of these experiments.

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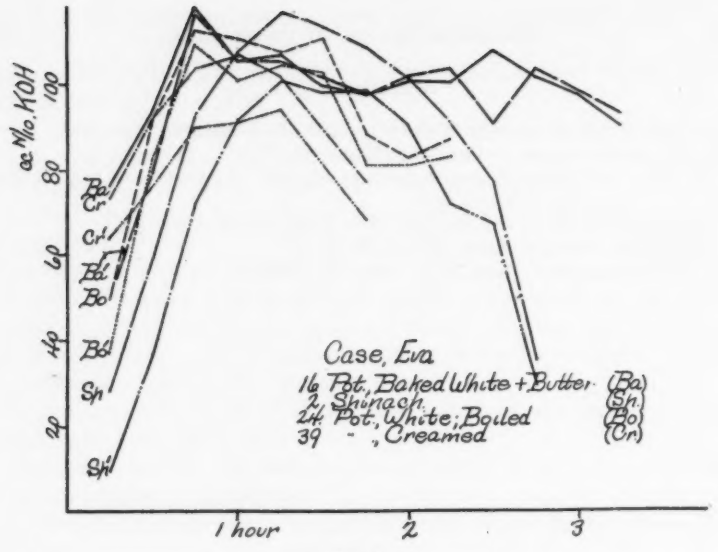
cc N/10 KOH
100
80
60
40
20
Ba
Cr
Cr'
Ba'
Bo
Bo'
Sp
Sp'
Case, Eva
16 Pot., Baked White + Butter (Ba)
2 Spinach (Sp)
24 Pot., White; Boiled (Bo)
39 " , Creamed (Cr)
1 hour
2
3

FIG. 1

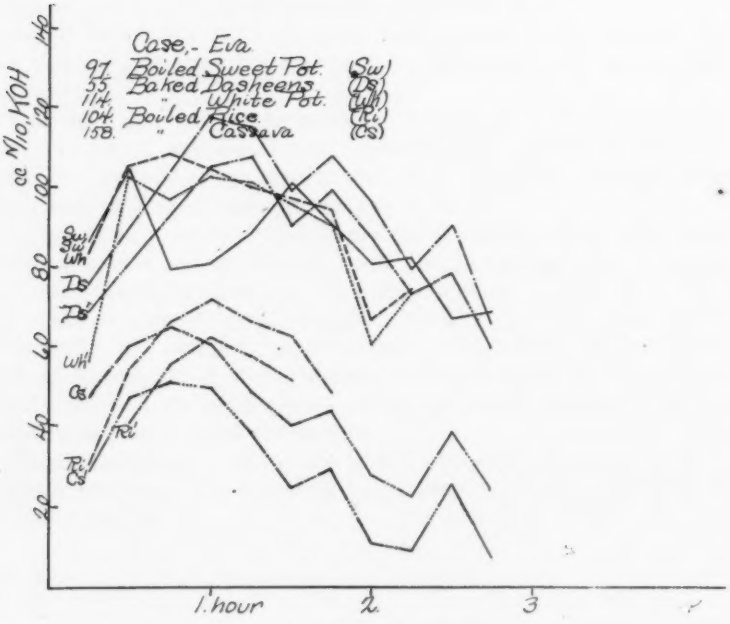
Case, - Eva
97 Boiled Sweet Pot. (Sw)
55 Baked Dasheens (Ds)
114 " White Pot. (Wh)
104 Boiled Rice (Rc)
158 " Cassava (Cs)
cc N/10 KOH
140
120
100
80
60
40
20
Sw
Sw'
Wh
Ds
Ds'
Wh'
Cs
Rc'
Rc
Cs'
1 hour
2
3

FIG. 2

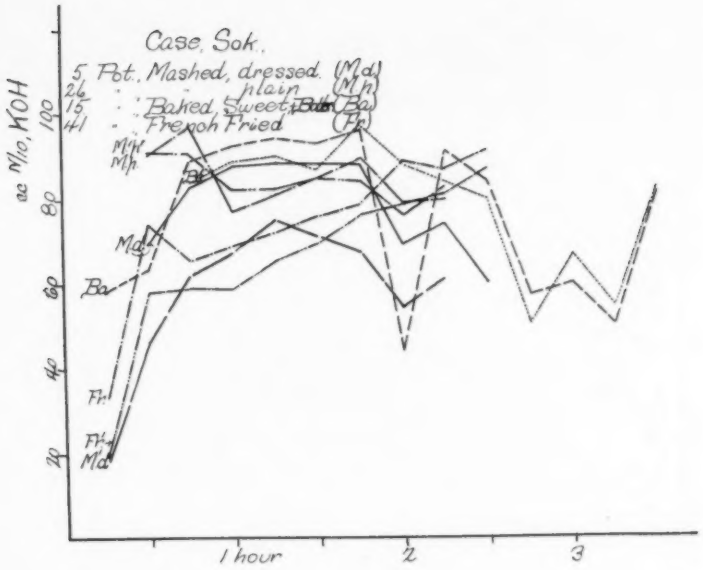
Case, Sok.
5 Pot. Mashed, dressed (Md)
24 " " plain (Mp)
15 " Baked, Sweet, Ba
41 " French Fried (Fr)
cc N/10, KOH
100
80
60
40
20
Mdr
Mp
Ba
Fr
Fr
Md
1 hour
2
3

FIG. 3

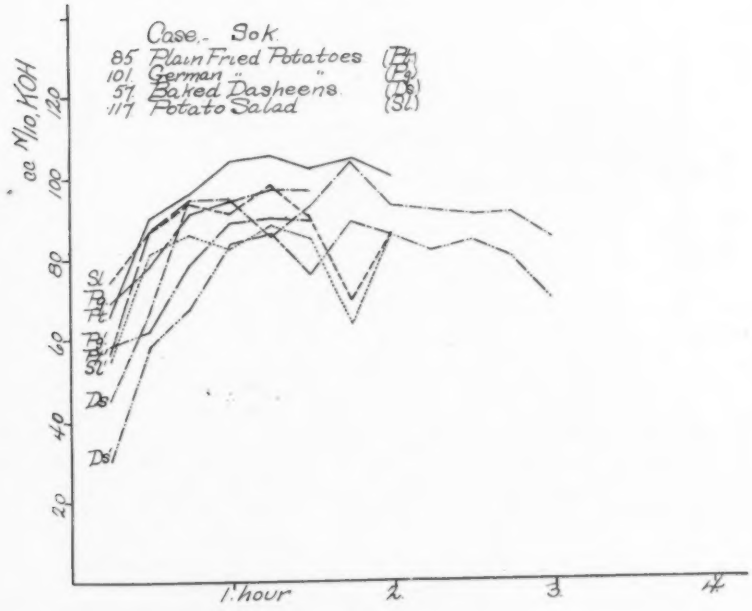
Case, Sok.
85 Plain Fried Potatoes (Pf)
101 German " " (Pg)
57 Baked Dasheens (Ds)
117 Potato Salad (Sl)
cc N/10, KOH
120
100
80
60
40
20
Sl
Pg
Pf
Pf
Sl
Ds
Ds
1 hour
2
3
4

FIG. 4

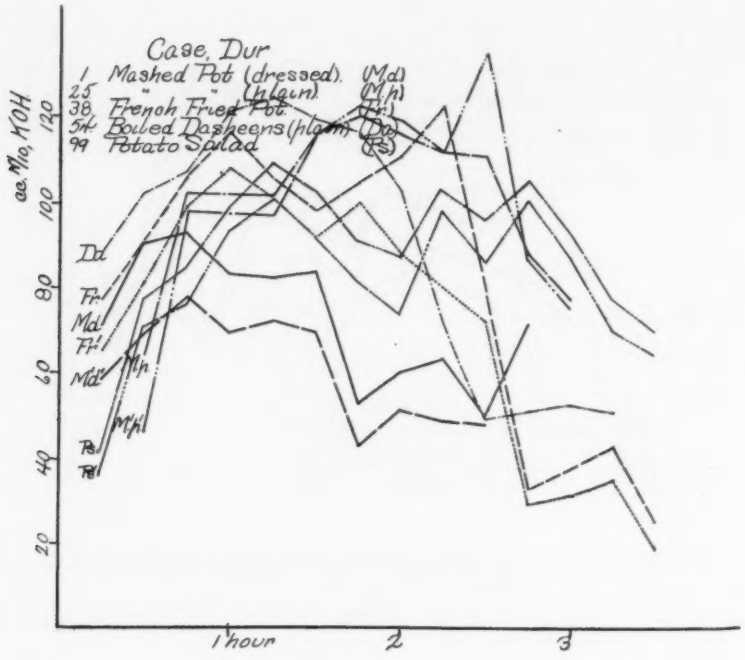
Case, Dur
1 Mashed Pot (dressed) (Md)
25 " (plain) (Mp)
38 French Fried Pot. (Fr)
54 Boiled Dasheens (plain) (Ds)
99 Potato Salad (Ps)
cc. N/10 KOH
120
100
80
60
40
20
Dd
Fr
Md
Fr'
Md'
Mp
Mp'
Ps
Ps'
1 hour
2
3

FIG. 5

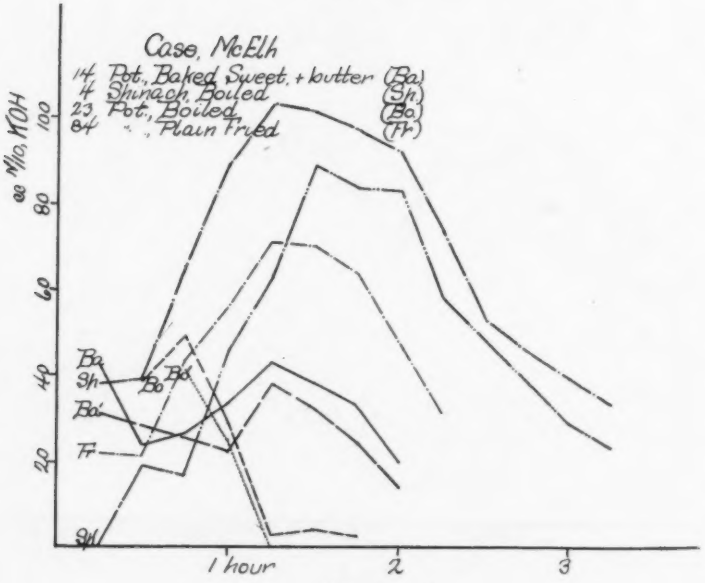
Case, McElh
14 Pot., Baked Sweet, + butter (Ba)
4 Spinach, Boiled (Sh)
23 Pot., Boiled (Bo)
84 " Plain Fried (Fr)
cc N/10 KOH
100
80
60
40
20
Ba
Sh
Bo
Bo'
Ba'
Fr
Sh'
1 hour
2
3

FIG. 6

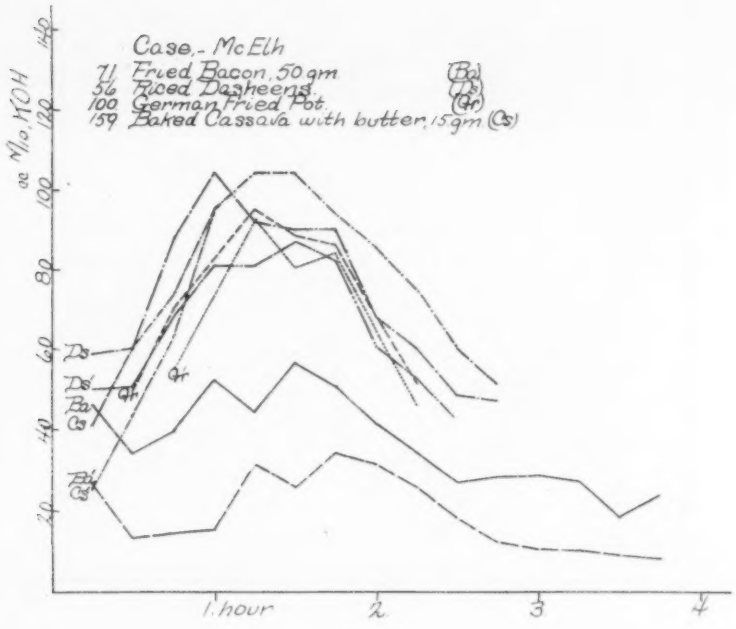
Case, - McElh
71 Fried Bacon, 50 gm (Ba)
56 Riced Dasheens (Ds)
100 German Fried Pot. (Gr)
159 Baked Cassava with butter, 15 gm (Cs)
cc N/10 KOH
1 hour
2
3
4

FIG. 7

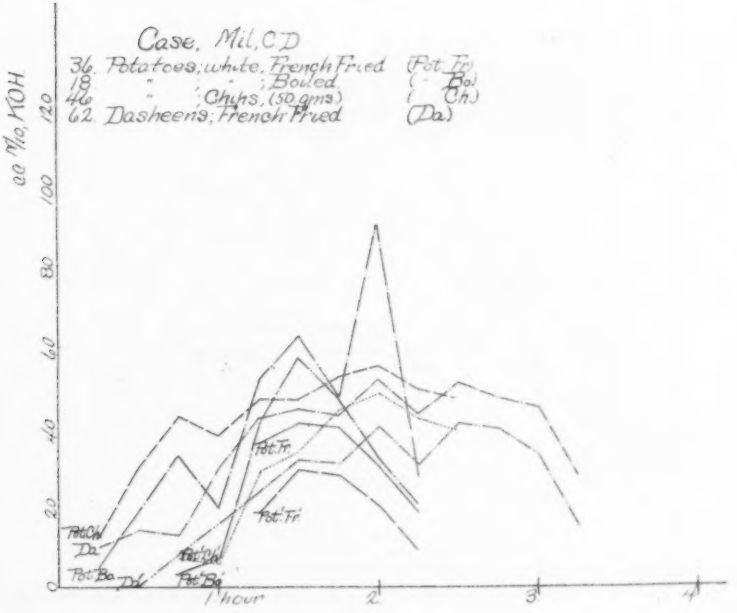
Case, Mil, C D
36. Potatoes, white, French Fried (Pot. Fr)
18 " " ; Boiled (" Bo)
46 " ; Chips, (50 gms) (" Ch)
62. Dasheens; French Fried (Da)
cc N/10 KOH
1 hour
2
3
4

FIG. 8

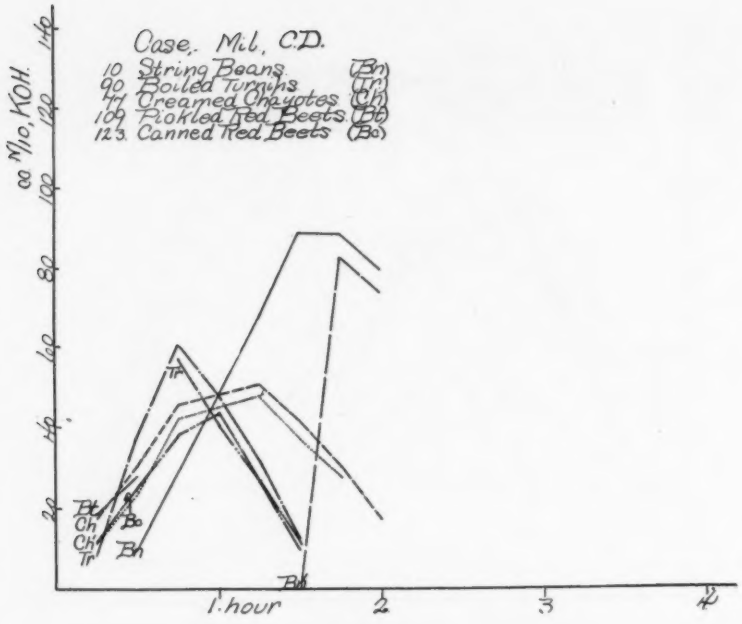
Case, Mil, C.D.
10 String Beans (Bn)
90 Boiled Turnips (Tr)
111 Creamed Chayotes (Ch)
169 Pickled Red Beets (Bb)
123 Canned Red Beets (Bo)
cc N/10 KOH
1 hour
2
3
4

FIG. 9

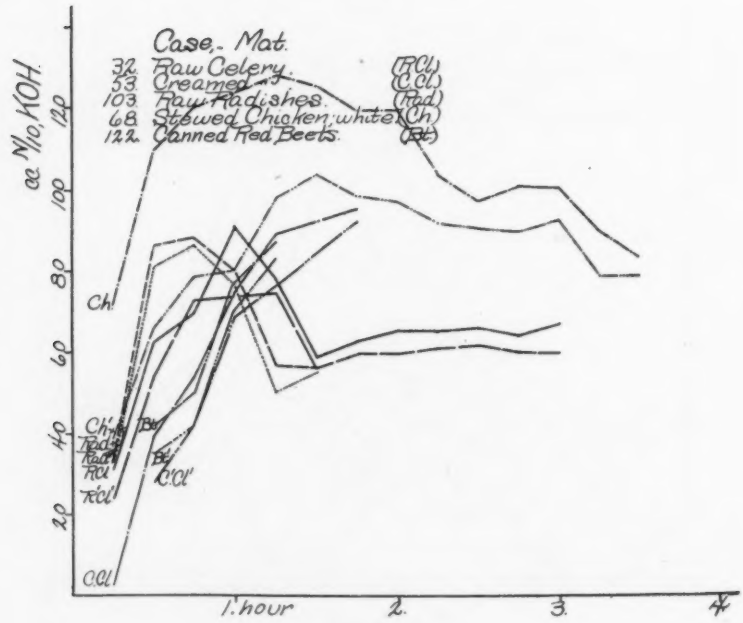
Case, Mat.
32 Raw Celery (RCl)
53 Creamed (CCl)
103 Raw Radishes (Rad)
68 Stewed Chicken white (Ch)
122 Canned Red Beets (Bo)
cc N/10 KOH
1 hour
2
3
4

FIG. 10

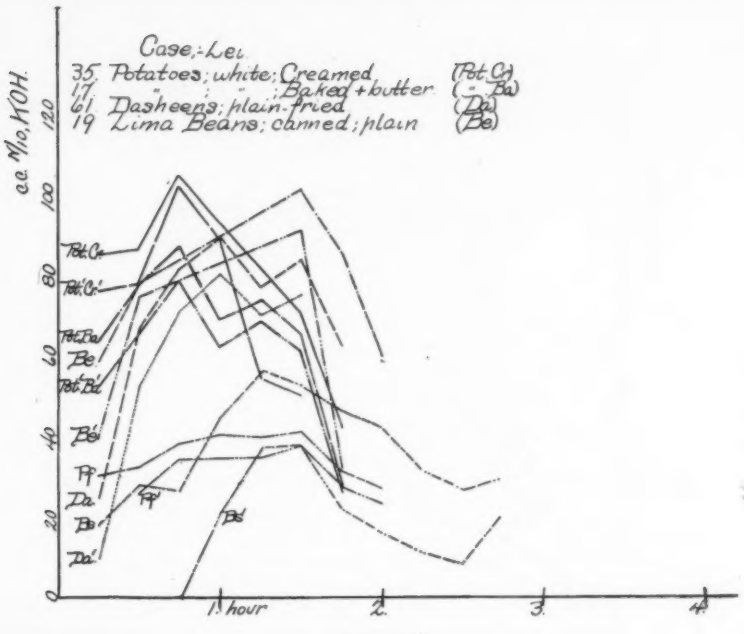
Case,- Lei
35 Potatoes; white; Creamed (Pot Cr)
17 " ; Baked + butter (" Bu)
61 Dasheens; plain-fried (Da)
19 Lima Beans; canned; plain (Be)
cc N/10 KOH
0
20
40
60
80
100
120
1 hour
2
3
4

FIG. 11

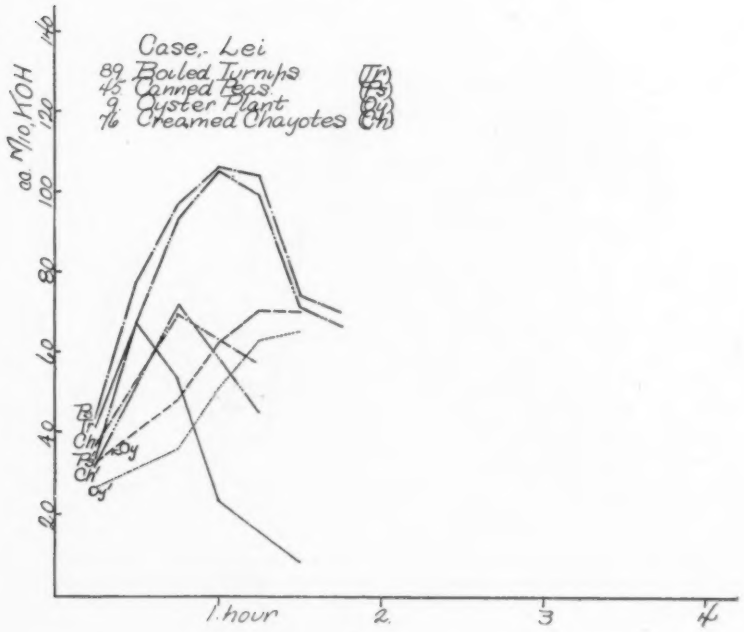
Case,- Lei
89 Boiled Turnips (Tr)
45 Canned Peas (Pe)
9 Oyster Plant (Oy)
76 Creamed Chayotes (Ch)
cc N/10 KOH
20
40
60
80
100
120
140
1 hour
2
3
4

FIG. 12

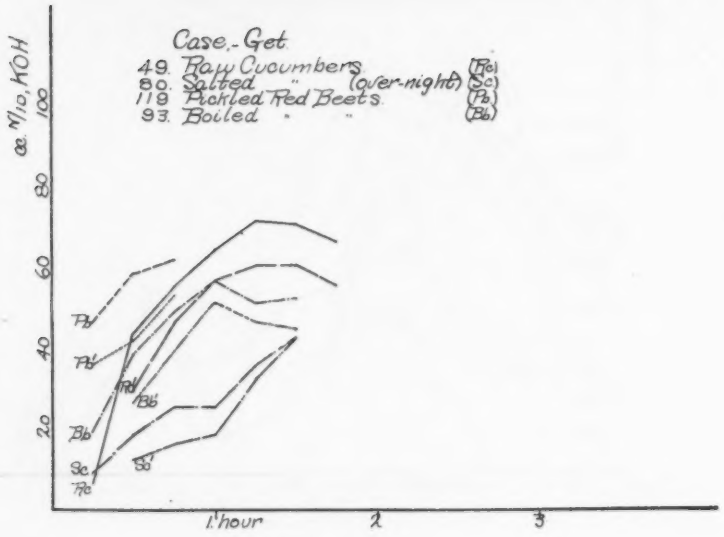
Case, - Get
49. Raw Cucumbers (Rc)
80. Salted " (over-night) (Sc)
119 Pickled Red Beets (Pb)
93. Boiled " " (Bb)
cc N/10, KOH
100
80
60
40
20
1 hour
2
3

FIG. 13

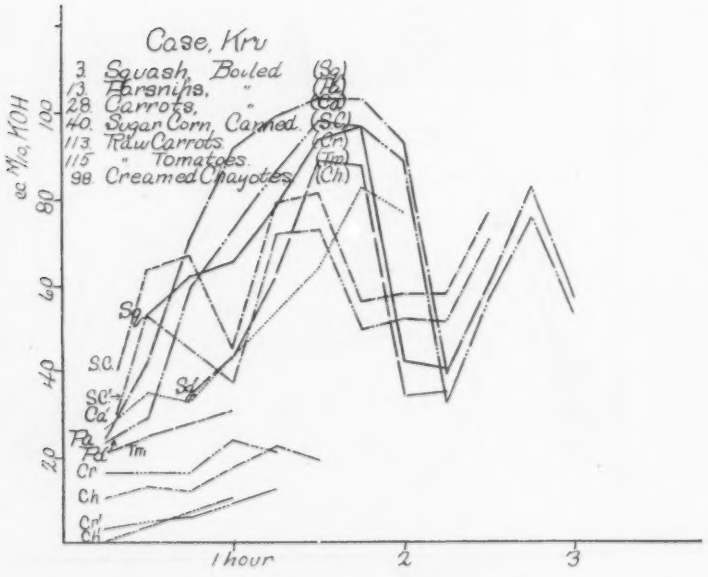
Case, Kru
3 Squash, Boiled (Sq)
13 Parsnips, " (Pa)
28 Carrots, " (Ca)
40. Sugar Corn, Canned (SC)
113. Raw Carrots (Cr)
115 " Tomatoes (Tm)
98 Creamed Chayotes (Ch)
cc N/10, KOH
100
80
60
40
20
1 hour
2
3

FIG. 14

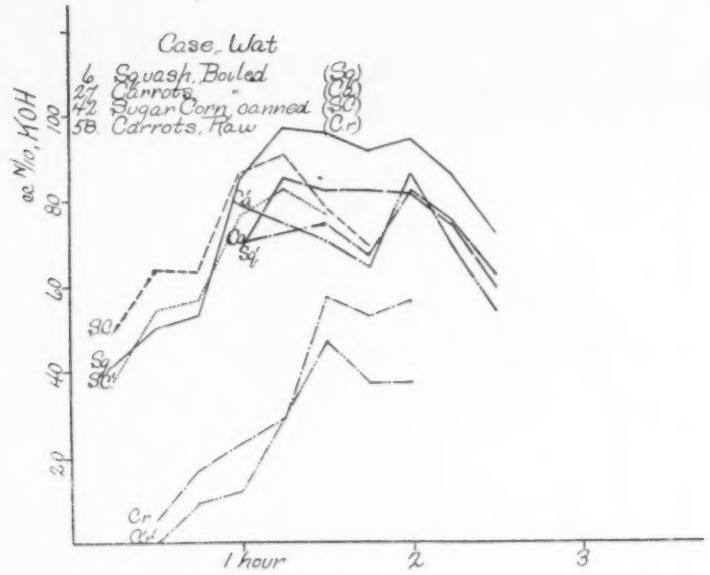
Case, Wat
6 Squash, Boiled (Sq)
27 Carrots " (Cr)
42 Sugar Corn, canned (Sc)
58 Carrots, Raw (Cr)
cc N/10 KOH
1 hour
2
3

FIG. 15

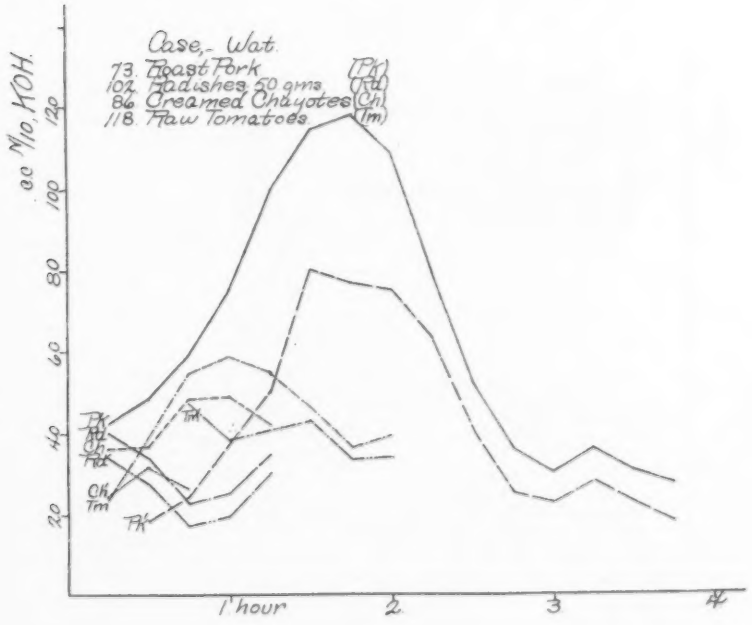
Case, Wat.
73 Roast Pork (Pk)
102 Radishes 50 grms (Rd)
86 Creamed Chayotes (Ch)
118 Raw Tomatoes (Tm)
cc N/10 KOH
1 hour
2
3
4

FIG. 16

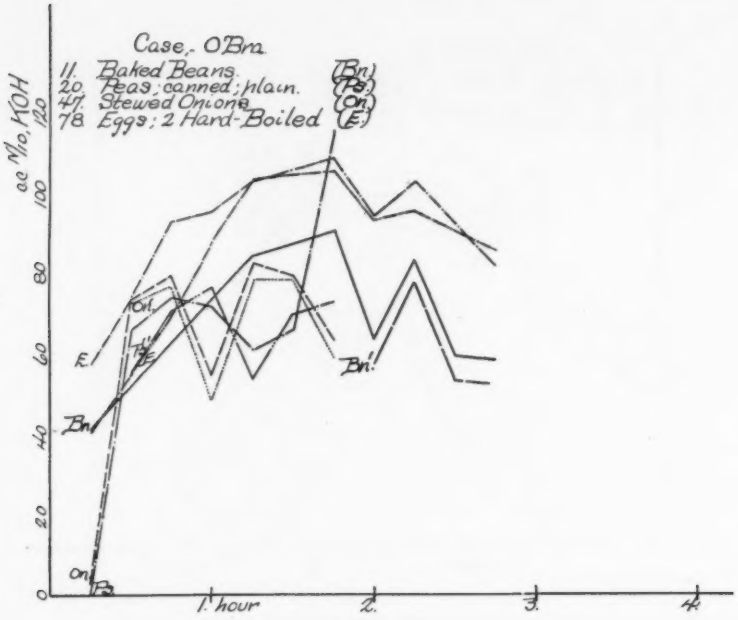
Case,- O'Brn.
11. Baked Beans (Bn)
20 Peas; canned; plain. (Pa)
47 Stewed Onions (On)
78 Eggs; 2 Hard-Boiled (E)
cc N/10 KOH
0
20
40
60
80
100
120
1 hour
2
3
4

FIG. 17

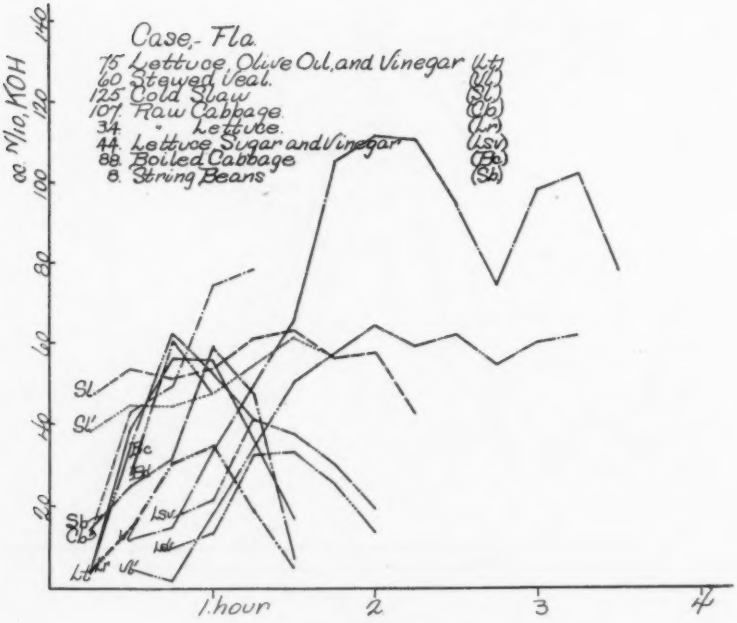
Case,- Fla.
75 Lettuce, Olive Oil, and Vinegar (Lt)
60 Stewed Veal. (V)
125 Cold Slaw (Sl)
107 Raw Cabbage (Cb)
34 " Lettuce. (Lr)
44 Lettuce, Sugar and Vinegar (LSV)
88 Boiled Cabbage (Bc)
8 String Beans (Sb)
cc N/10 KOH
0
20
40
60
80
100
120
140
1 hour
2
3
4

FIG. 18

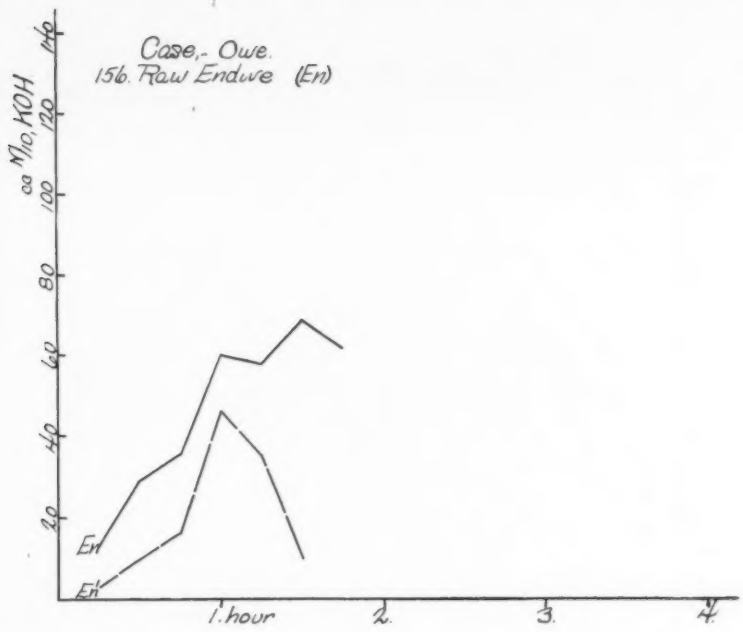
Case,- Owe.
156. Raw Endive (En)
cc N/10 KOH
140
120
100
80
60
40
20
En
En'
1. hour
2.
3.
4.

FIG. 19

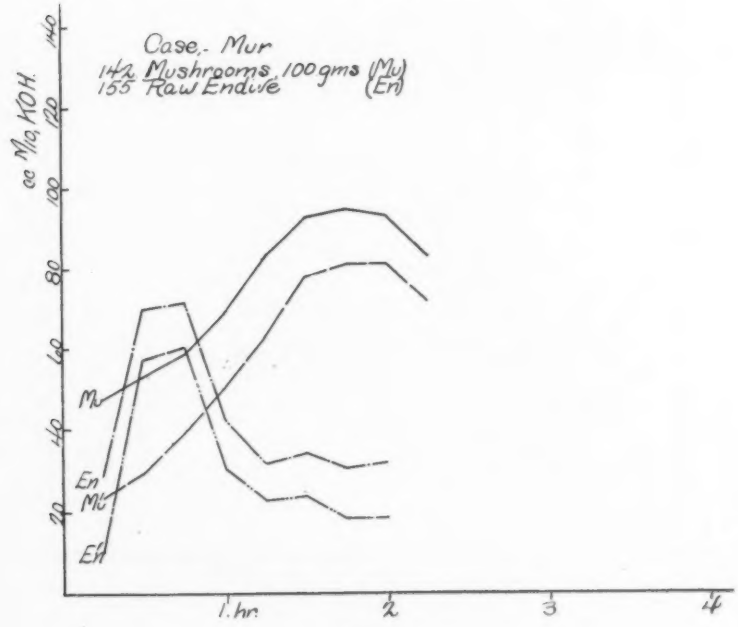
Case,- Mur
142 Mushrooms, 100 gms (Mu)
155 Raw Endive (En)
cc N/10 KOH
140
120
100
80
60
40
20
Mu
En
Mu'
En'
1. hr.
2
3
4

FIG. 20

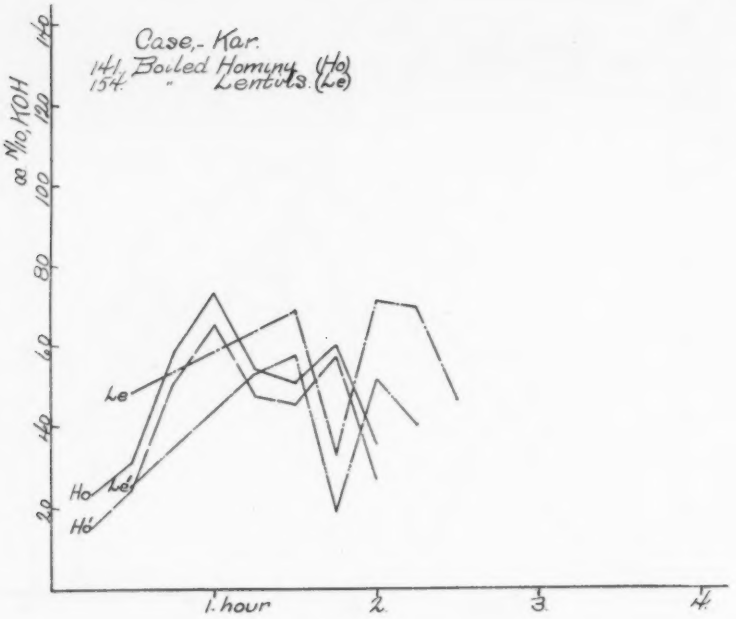
Case,- Kar.
141 Boiled Hominy (Ho)
154 " Lentils (Le)
cc N/10 KOH
140
120
100
80
60
40
20
Le
Ho
Le'
Ho'
1. hour
2
3
4

FIG. 21

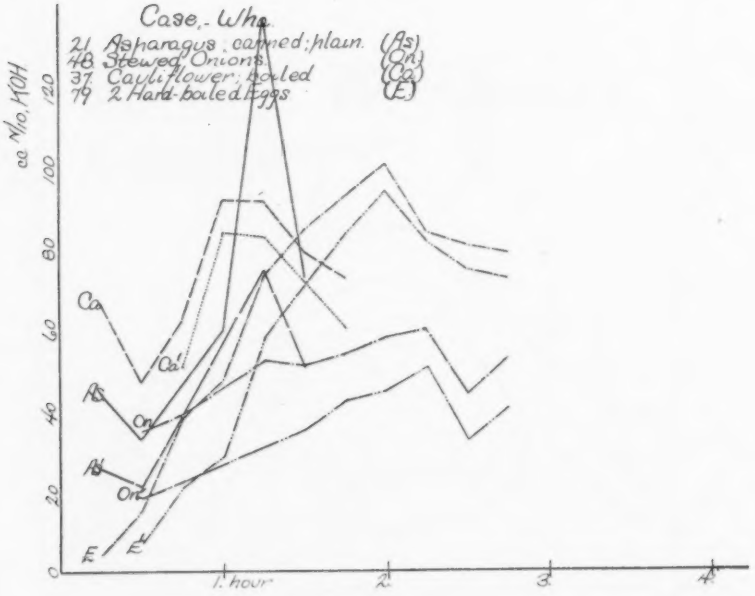
Case,- Whe
21 Asparagus, canned; plain (As)
48 Stewed Onions (On)
37 Cauliflower; boiled (Ca)
79 2 Hard-boiled Eggs (E)
cc N/10 KOH
120
100
80
60
40
20
0
Ca
Ca'
As
On
As'
On'
E
E'
1. hour
2
3
4

FIG. 22

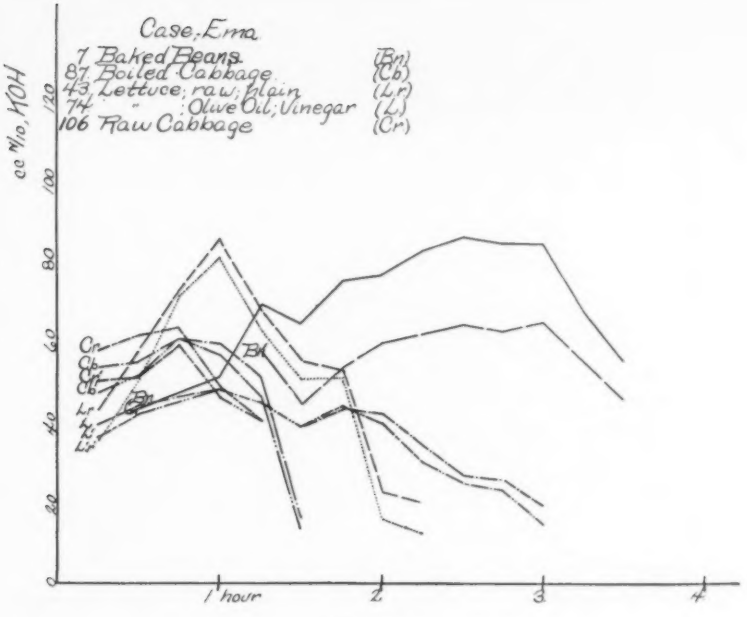
Case, Erna
7 Baked Beans (Bn)
87 Boiled Cabbage (Cb)
43 Lettuce; raw; plain (Lr)
74 " ; Olive Oil; Vinegar (L)
106 Raw Cabbage (Cr)
cc N/10 KOH
0
20
40
60
80
100
120
1 hour
2
3
4

FIG. 23

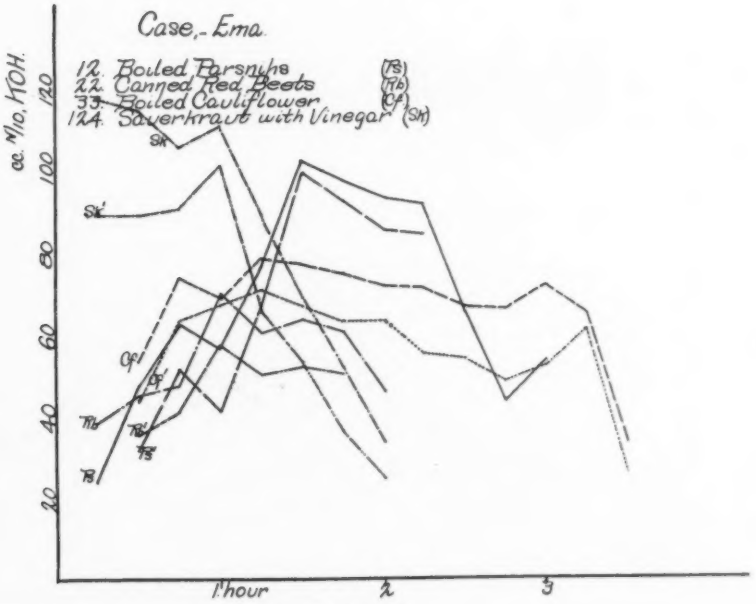
Case, Erna
12 Boiled Parsnips (Ps)
22 Canned Red Beets (Rb)
33 Boiled Cauliflower (Cf)
124 Sauerkraut with Vinegar (Sk)
cc N/10 KOH
20
40
60
80
100
120
1 hour
2
3

FIG. 24

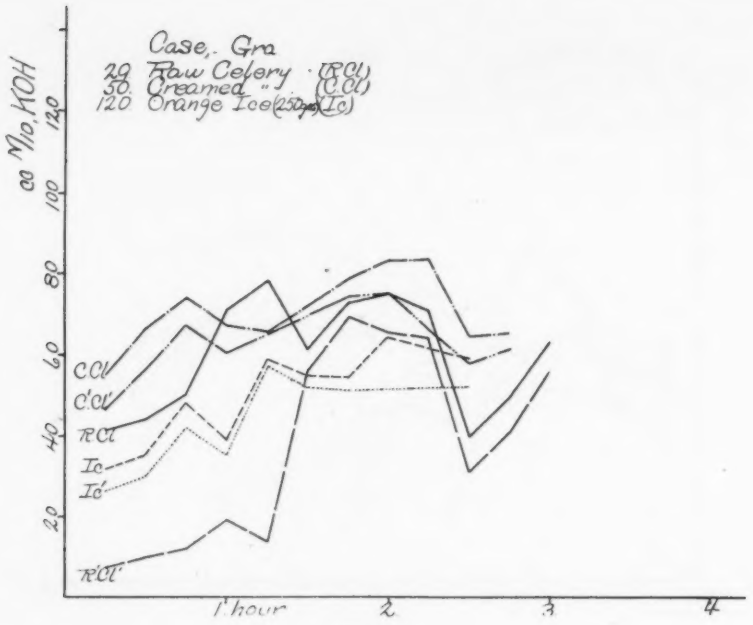
Case, Gra
29 Raw Celery (RCl)
30 Creamed " (CCl)
120 Orange Ice (Ic)
cc N/10, KOH
120
100
80
60
40
20
CCl
C'Cl'
RCl
Ic
Ic'
RCl'
1 hour
2
3
4

FIG. 25

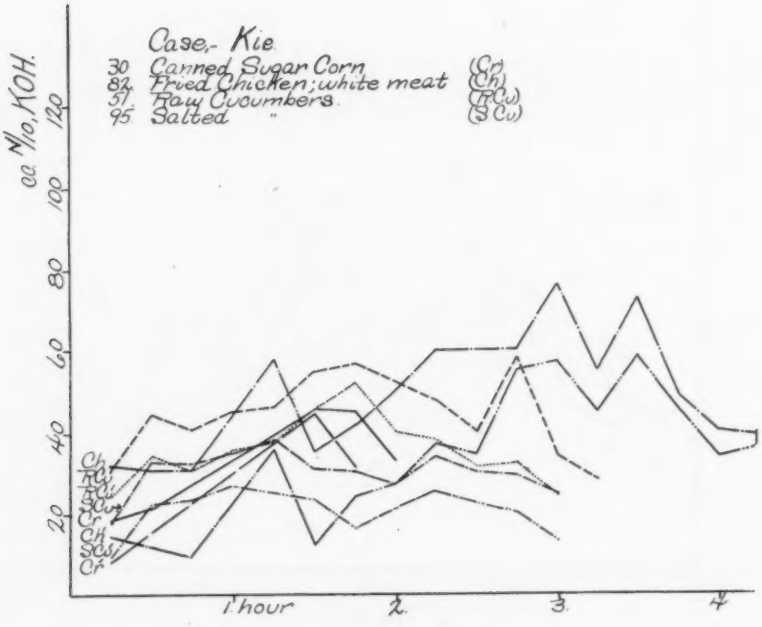
Case, Kie
30 Canned Sugar Corn (Cr)
82 Fried Chicken; white meat (Ch)
51 Raw Cucumbers (RCu)
95 Salted " (SCu)
cc N/10, KOH
120
100
80
60
40
20
Ch
RCu
RCu'
SCu
Cr
Ch'
SCu'
Cr'
1 hour
2
3
4

FIG. 26

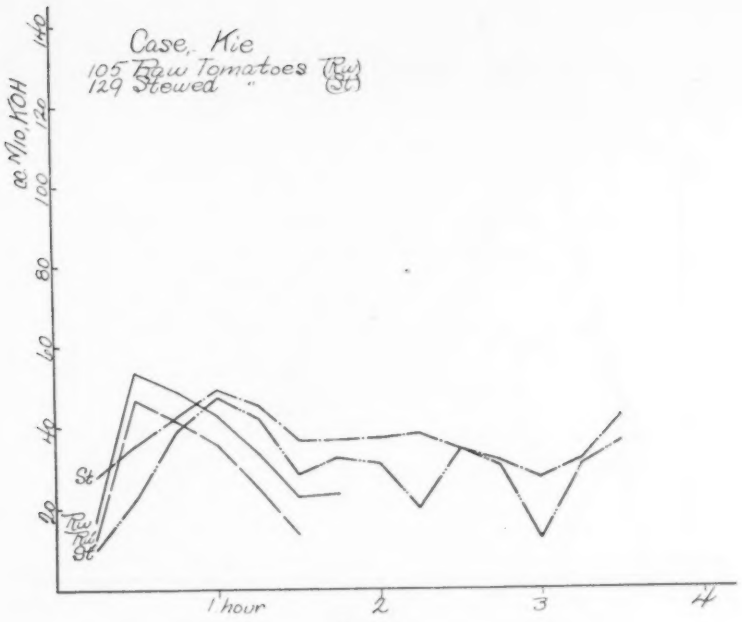
Case, Kie
105 Raw Tomatoes (Rw)
129 Stewed " (St)
cc N/10 KOH
140
120
100
80
60
40
20
St
Rw
Rw
St
1 hour
2
3
4

FIG. 27

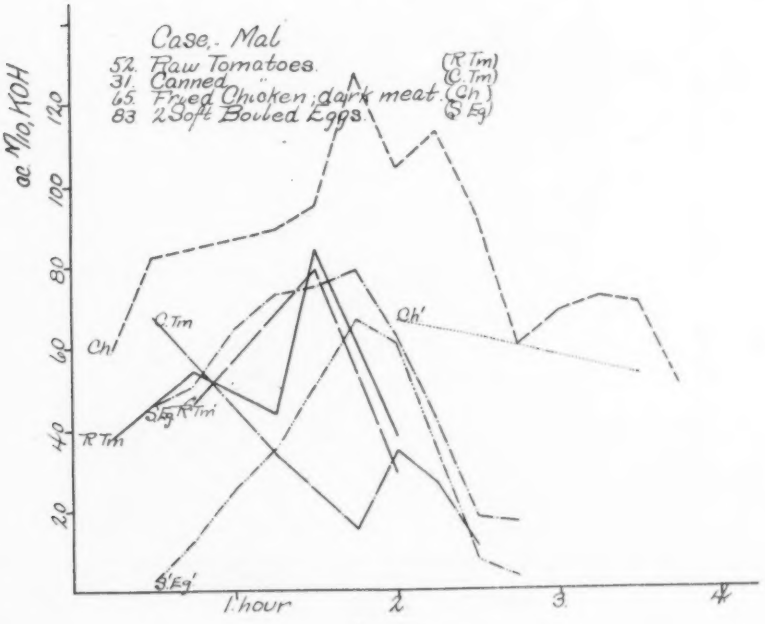
Case, Mal
52 Raw Tomatoes (R Tm)
31 Canned " (C Tm)
65 Fryed Chicken, dark meat (Ch)
83 2 Soft Boiled Eggs (S Eg)
cc N/10 KOH
120
100
80
60
40
20
Ch
C Tm
Ch'
S Eg
R Tm
R Tm
S'Eg'
1 hour
2
3
4

FIG. 28

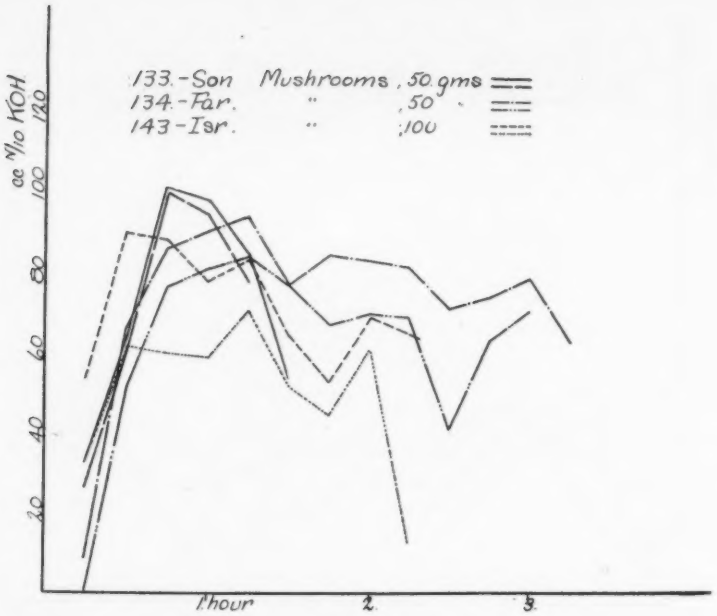
133.-Son Mushrooms, 50 gms
134.-Far. " ,50
143.-Isr. " ;100
cc N/10 KOH
120
100
80
60
40
20
1hour
2.
3.

FIG. 29

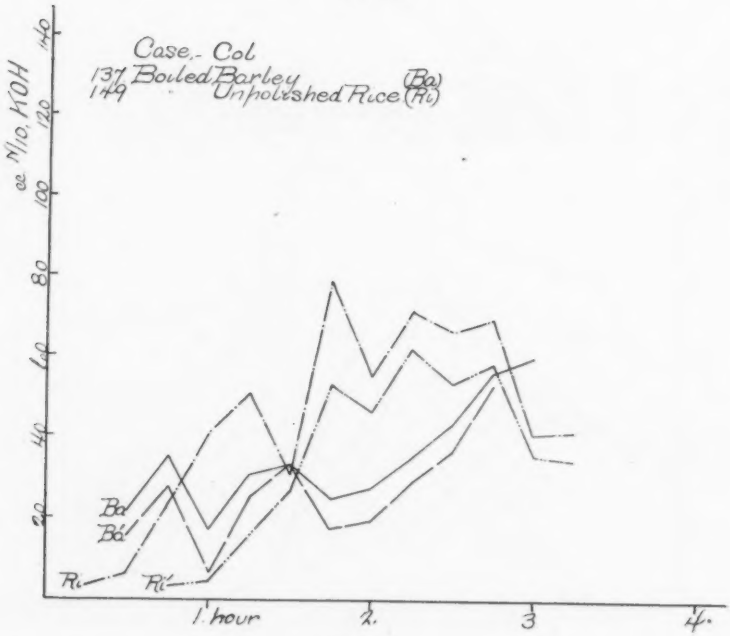
Case.- Col
137 Boiled Barley (Ba)
149 Unpolished Rice (Ri)
cc N/10 KOH
140
120
100
80
60
40
20
Ba
Ba'
Ri
Ri'
1. hour
2.
3
4.

FIG. 30

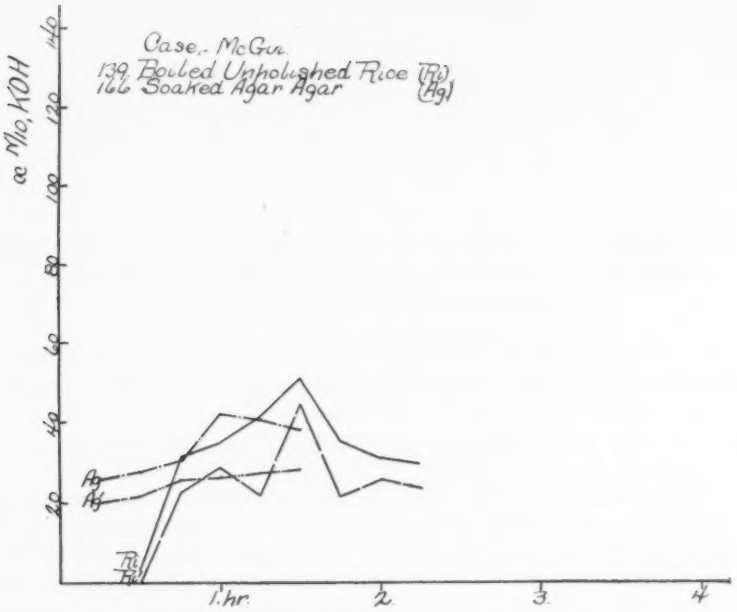
Case. McGu
139 Boiled Unpolished Rice (Ri)
166 Soaked Agar Agar (Ag)
cc N/10 KOH
Ag
Ag
Ri
Ri
1 hr.
2
3
4

FIG. 31

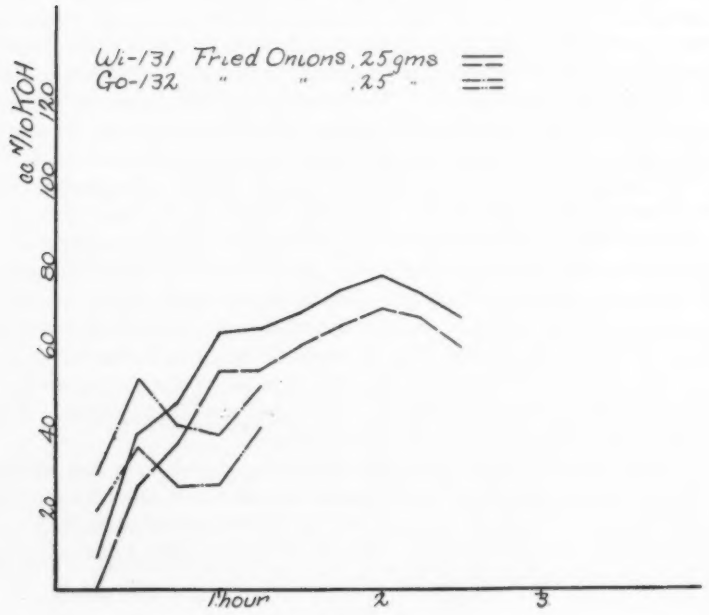
Wi-131 Fried Onions, 25 gms
Go-132 " " .25 "
cc N/10 KOH
1 hour
2
3

FIG. 32

FURTHER OBSERVATIONS ON THE RELATION OF THE ADRENALS TO CERTAIN EXPERIMENTAL HYPERGLYCEMIAS (ETHER AND ASPHYXIA)

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The question whether the epinephrin output of the adrenals is essentially concerned in the production of certain experimental hyperglycemias has been much debated. Two main ways of approaching the question are theoretically open: *a*, the estimation of the output of epinephrin under the action of the factors inducing the experimental hyperglycemia, in order to show whether this can be sufficiently great to permit the introduction into the blood of the necessary excess of epinephrin as determined by artificial injection; *b*, study of the blood sugar content in the absence of the adrenals or after interference with their epinephrin secretion, when the conditions which cause hyperglycemia with intact adrenals are induced. The first method of approach might seem to be the more direct, but it has been little used. Such results as have been obtained on the rate of output of epinephrin in ether narcosis and asphyxia are quite unfavorable to the view that epinephrin plays any sensible rôle in the production of the hyperglycemia.

Of the numerous researches made by the second method many, unfortunately, however interesting in other relations, throw no light upon the question at issue because essential conditions were not fulfilled. The most important of these seem to be the following:

1. The glycogen store must be adequate to permit of a decided hyperglycemia. Negative results cannot otherwise be accepted. The only sure way is to estimate the liver glycogen.

2. Only animals which have completely recovered from the effects of the operation practised to eliminate the adrenal epinephrin output should be used. Cats as employed by us, after removal of one adrenal and section of the nerves of the other, and rabbits which have survived double adrenalectomy, fulfil this condition.

3. The question of the rôle of the adrenals should not in the present state of our knowledge be complicated by unnecessary interference with the nerve supply of the liver. For example, negative results after section of both splanchnics cannot be used to determine the question of the indispensability of the adrenals. In our experiments on ether, asphyxia (1) and piqûre hyperglycemia (2) only one splanchnic was cut in the cats and neither splanchnic in the rabbits.

We believe it is inadvisable to complicate the question whether the adrenals are essential for the development of experimental hyperglycemias with speculations on the possible output of epinephrin from the diffuse chromaffine tissue. There is no evidence as to the possible magnitude of the output of epinephrin, if there is any output, from these scattered chromaffine cells and no obvious way of investigating the matter.[1] Our own experiments have been concerned solely with the adrenals. Whether they are essential for the hyperglycemias studied is a question which can be definitely settled. We believe it has been definitely decided in the negative, and that the so-called "adrenalin hypothesis" should be abandoned, at least in the case of these forms of hyperglycemia.

Recently, however, Keeton and Ross (3) have published a paper on the mechanism of ether hyperglycemia in dogs, in which they make the point that a short etherization is associated with some hyperglycemia even when both splanchnic nerves have been cut, but that this does not persist, under continuous insufflation of ether, as it does in the case of normal dogs. Incidentally they raise the question whether the etherization in our experiments was continued long enough. They think it was not, and that we might have obtained a different result with a longer period of etherization. For this reason they do not consider that we have demonstrated conclusively that ether hyperglycemia can occur in the absence of the epinephrin output of the adrenals. Section of both splanchnics introduces the complication that the innervation of the liver is greatly interfered with, whereas our object was to interfere only with the adrenals, and in this regard

[1] If the diffuse chromaffine tissue is under the control of nerves in respect of any output of epinephrin, as is to be supposed since it represents sympathetic nerve cells, the output could be greatly interfered with, it may be assumed, by section of sympathetic fibers, including the splanchnics, or by appropriate spinal cord section. In animals which have survived in good health some of the operations practised by us, it seems probable that a large part of the epinephrin output, if any exists, of the diffuse chromaffine tissue was suppressed as well as that of the adrenals.

our experiments are not really comparable with those of Keeton and Ross. Nevertheless, as courteous criticism is always helpful and as, of course, it might be possible that a longer period of etherization should develop a difference between normal animals and those in which the adrenal epinephrin output has been interfered with, we have made some experiments in which the blood sugar was determined at the end of a short period (20 minutes) and again at the end of a much longer period of etherization (80 to 90 minutes). The experiment was wound up with a period of asphyxia and another blood sample collected, since asphyxia is one of the surest methods of producing hyperglycemia, and in the event of a negative result with ether the asphyxia sample serves as a control to show whether the nutritive condition of the animal, especially the glycogen store, was compatible with a well marked hyperglycemia. In addition, the glycogen content of the liver was estimated.

Before proceeding to these experiments, however, it seems necessary to explain what we aimed at in our previous experiments. Keeton and Ross state that "of four of their (Stewart and Rogoff's) experiments, only two show a gain (0.087 to 0.151 and 0.142 to 0.176) that does not fall within the limit of experimental error. The other two (0.200 to 0.233 and 0.092 to 0.098) are not significant." This criticism is based on a misapprehension. We made a number of blood sugar experiments on each cat at different dates. In some instances the results were negative in one of the experiments, while on another day, perhaps a week or more thereafter, the animal meanwhile being specially fed, the results were positive, doubtless owing to the difference in the nutritional state (glycogen content of liver), a condition which is known to be of the highest importance for the production of such experimental hyperglycemias. Precisely similar results are obtained in normal animals.

In the new experiments rabbits which had survived the removal of both adrenals were employed, and here of course there was no question of any residual liberation of epinephrin from the adrenals. The animals had perfectly recovered from the primary operations, and for 8 to 11 days before the blood sugar tests were placed on a diet of carrots in addition to the routine diet of oats and hay, and sugar was added to the drinking water twice a day. The glycogen content of the liver was estimated at the end of the experiment. The results, as will be seen from the following protocols, were entirely confirmatory of our results on cats.

Condensed protocol. Rabbit 409

September 19, 1919. Excised right adrenal.

October 21, 1919. Excised left adrenal. Weight of rabbit 2.51 kgm.

November 17 to November 25, 1919. Carrots in addition to usual diet (oats and hay) and cane sugar added to drinking water, twice daily.

November 25, 1919. Condition excellent. Weight 2.67 kgm.

9. 30 a.m. 2 cc. blood (from ear vein) contained 0.13 per cent dextrose.

9.40 a.m. Started light etherization.

10.05 a.m. 2 cc. blood (from ear vein) contained 0.16 per cent dextrose. Continued light etherization for an hour longer.

11.10 a.m. 2 cc. blood (from ear vein) contained 0.27 per cent dextrose. Now discontinued etherization.

11.25 a.m. Started asphyxia and continued it intermittently till

11.45 a.m. 2 cc. blood (from ear vein) contained 0.37 per cent dextrose.

The liver, excised and hydrolyzed at once, contained 3.13 per cent of glycogen. The liver weighed 40.3 grams.

Of course, the glycogen content at the beginning of the experiment would be decidedly greater than that estimated at the end.

Condensed Protocol. Rabbit 410

September 19, 1919. Excised right adrenal.

October 21, 1919. Excised left adrenal. Weight of rabbit 3.18 kgm.

November 17 to November 28, 1919. Carrots in addition to usual diet (oats and hay) and cane sugar added to drinking water, twice daily.

November 28, 1919. Condition excellent. Weight 3.25 kgm.

9.30 a.m. 2 cc. blood (from ear vein) contained 0.11 per cent dextrose.

9.40 a.m. Started etherization (to surgical anesthesia).

10.00 a.m. 2 cc. blood (from ear vein) contained 0.27 per cent dextrose. Continued etherization (lightly) for an hour longer.

11.05 a.m. 2 cc. blood (from ear vein) contained 0.41 per cent dextrose. Now discontinued etherization.

11.15 a.m. Started asphyxia: after 7 minutes of asphyxia (with a towel over nose) applied off and on (being guided by the slowing of the heart rate) the circulation became poor and the respirations shallow and slow. Artificial respiration was started and an attempt made to obtain blood from the femoral vein, but failed, owing to the poor blood flow. The chest was opened and 2 cc. of blood were obtained from the right heart. The heart was beating feebly and the blood was dark. This specimen contained 0.53 per cent dextrose.

The liver was excised 4 minutes after the last specimen was obtained and hydrolyzed at once. It contained 2.5 per cent of glycogen at the end of the experiment. At the beginning the glycogen content must have been greater. The surplus sugar in the blood at the end would alone correspond to an additional amount of 0.7 per cent glycogen in the liver.

Our results on asphyxial hyperglycemia (1), (2) both in cats after interference with the adrenal epinephrin output and in rabbits after

removal of both adrenals are quite as conclusive as those on ether hyperglycemia. Where the liver was well filled with glycogen a marked hyperglycemia was invariably obtained. What interpretation can possibly be placed upon such results except that the adrenal epinephrin is not essential to the production of these hyperglycemias?

Yet a quite recent writer, Yamakami (4), seems to think that direct evidence of this kind can be set aside because he has, as he supposes, shown that asphyxial blood, obtained from one normal rabbit and injected into another normal rabbit causes a rise in the sugar content of the blood. Even if the results which he quotes be accepted as proving a distinct augmentation in the blood sugar of the recipient rabbit, in addition to any increase due to the sugar actually present in the injected asphyxial blood (and not more than half of the experiments reproduced in his table could be considered positive), they are irrelevant to the question of the rôle of the adrenals in asphyxial hyperglycemia. They do not warrant "the hypothesis that adrenalin in the asphyxial blood is responsible because we do not know at present any other substance than adrenalin in the blood which can give rise to the enhanced sugar content." He endeavors to exclude changes in carbon dioxide content and possible changes in H-ion concentration in the asphyxial blood as factors. It would seem a more crucial test to estimate the epinephrin in the blood injected, since it is universally acknowledged that a certain amount of epinephrin will cause hyperglycemia. Underhill (5) showed that very large quantities of adrenalin introduced by continuous intravenous infusion into non-anesthetised rabbits (as much as 333 cc. of a 1:250,000 solution) did not cause glycosuria. It must be noted that the heart blood was taken from nearly dead, or in half the cases, from actually dead rabbits. With the extreme slowing of the blood flow in the inferior cava the concentration of epinephrin in the heart blood, so long as the adrenal epinephrin output was not affected, would tend to rise. The Japanese author seems to have had some suspicion that the adrenalin hypothesis might not have much to support it, for he continues "of course, we cannot venture to claim that hyperadrenalinemia was proved by our experiments to exist in asphyxia." Curiously enough he states early in the paper that "in order to solve the problem whether the adrenals are involved in the asphyxial hyperglycemia it seems to be the wisest method to study this hyperglycemia in animals whose adrenals were removed entirely," and he suggests rabbits which have survived double adrenalectomy as the most suitable. But he makes no mention of our

observations on piqûre and asphyxial hyperglycemia in such rabbits, which demonstrate that the adrenals are unnecessary. Nor does he utilize in his own observations what would be a really crucial experiment, the injection of asphyxial blood from an adrenalectomized rabbit into a normal rabbit, and into another adrenalectomized rabbit. He seems to confuse our method of collecting adrenal vein blood with the method of collecting specimens for blood sugar estimations. We did not of course anesthetize the animals, perform a laparotomy, etc., to obtain blood specimens when we were determining whether asphyxia could cause hyperglycemia in the absence of epinephrin discharge from the adrenals.

Kellaway (6) has recently verified our conclusion that the adrenal epinephrin output is not essential to the production of the hyperglycemia induced by asphyxia. He states that we have "denied that the suprarenals play any part in producing the hyperglycemia." We have not as a matter of fact put our conclusion in so absolute a form because, although our observations do not reveal any essential difference between normal animals and animals with the epinephrin output interfered with, in the degree of the hyperglycemia or the ease and certainty with which it is induced, it would be very difficult in experiments of this type to bring out clearly a small quantitative difference if such existed. Kellaway's blood sugar results in normal cats and in cats after interference with the adrenal epinephrin output are very much like our own, despite the fact that in his observations both splanchnics were divided and the innervation of the liver crippled.

His contention that increased adrenalin output is a factor in the hyperglycemia is not supported by his results so far as we can see. In most of his experiments he gets a good hyperglycemia with anoxemia (or asphyxia) after interference with the epinephrin output, exactly as we found. He has not estimated the liver glycogen in any of his animals nor does he indicate anywhere that he realizes its importance. He seems to assume that "the value of the threshold for anoxemia as regards hyperglycemia" can be fixed once for all for a given animal and that if breathing a certain percentage of oxygen causes hyperglycemia days or weeks before section of the splanchnics or removal of the adrenals, and fails to cause it after those operations, the difference must necessarily be due to the absence of epinephrin. It is impossble to accept the conclusion that because in one cat (exper. 14) "there was a very close correspondence between the production of a pupillary paradox and a rise in blood sugar" before section of the splanchnics and removal of

the adrenals, and because both reactions were absent or greatly reduced after these operations "it seems evident that in this cat the blood sugar effects were largely due to adrenalin." The failure to obtain hyperglycemia at the end of the experiment on the day after removal of both adrenals proves nothing at all, especially in the absence of any check on the glycogen store. The isolated observation (in exper. 15) that the intravenous injection of a quantity of adrenalin (0.1 cc. adrenalin 1 in 200,000) which was inadequate to elicit the paradoxical pupil reaction yet caused a hyperglycemia, is cited as further evidence that in asphyxial hyperglycemia adrenalin plays an important part. There was already a hyperglycemia present on account of the subjection of the animal to anoxemia but the apparent slight increase in blood sugar in the specimen taken after injection of the adrenalin is definitely attributed to the 0.0005 mgm. of adrenalin injected.

Kellaway's acceptance of the statements in the literature that asphyxia increases the rate of output of epinephrin apparently accounts for the uncritical way in which he finds support in his own observations for the view that increased adrenalin output is a considerable factor in asphyxial hyperglycemia. He seems to forget that it would not be enough to prove that asphyxia causes an increase in the epinephrin output, it must be shown that the increase is sufficient to bring the epinephrin content of the blood to the level necessary for adrenalin hyperglycemia and to maintain it there.

In our own work (7), (8), (9), we have not found evidence that there is any detectable increase in the output per unit of time, although, of course, when the blood flow through the adrenals is slowed the concentration of epinephrin in it is increased. The reason why our result differs from that of Kellaway is that we used quantitative methods which really enabled us to measure the output of epinephrin whereas he did not make any measurements at all, but assumed from the effect of asphyxia in causing the paradoxical pupillary reaction that there must have been an increase in the output. This is not a specific qualitative reaction for adrenalin, let alone a reaction by which the rate of output can be quantitatively estimated.[2] The fundamental difference

[2] The pupil reaction can be utilized for estimating quantitatively the epinephrin in adrenal vein blood, and it has been so employed by us. But to do this variations in the other factors which may affect the pupil (anesthesia, asphyxia, etc.) must be eliminated, the only change made being the addition to the general blood of the epinephrin containing-adrenal blood collected in a cava pocket for a definite time or the adrenalin artificially injected to assay it.

between Doctor Kellaway's work and our own embraces much more than the single question of the effect of asphyxia on the epinephrin output. All our work on the influence of various conditions and various substances upon the epinephrin output has aimed at a quantitative determination of epinephrin in the adrenal vein blood. Being able to tell how much epinephrin the adrenals were giving off per minute before the factor under investigation was allowed to act, and how much they were giving off while it was acting, we were naturally in a different position for determining whether any change had occurred from that occupied by an observer who could not have any idea how much epinephrin was being given off at any time throughout his experiment.

He has shown that the anoxemia is the important factor in producing the paradoxical diltaation of the pupil in asphyxia. This is a new and interesting point. By graduating the severity of the asphyxia, as he terms it, i.e., by causing the animal to breathe mixtures with definite percentages of oxygen less than that of the atmosphere, he has satisfied himself that after interference with the epinephrin output it is less easy to provoke the paradoxical dilatation than in normal animals, although in non-anesthetized animals he still gets a fair reaction. He studied the difference produced in this reaction in cats by section of the splanchnics in survival experiments and by removal of both adrenals. We have no observations on cats after bilateral splanchnotomy. Our animals were prepared by excision of one adrenal and section of the nerves of the other according to Elliott's method, and we were unable to convince ourselves, as we have stated in a previous paper (10) "that there is any striking difference" in the paradoxical reaction induced by asphyxia in these animals as compared with normal cats, although "we should rather expect a difference if the normal epinephrin output," as we believe, "is already exerting an action" on the sensitised iris. Kellaway also obtained a relatively small difference between the normal and operated cats with the severer grades of asphyxia, and it is possible that by using his "graduated" method instead of the cruder methods previously employed a similar difference would be made out in cats prepared in the way mentioned as he found in cats after section of the splanchnics. But this, so far as we can see, would indicate merely that the epinephrin was a factor in the asphyxial paradoxical reaction and would not prove that the output was *augmented* by asphyxia.

We have obtained evidence (10) that epinephrin passing into the blood at the ordinary rate under the conditions of our experiments exerts an action on the pupil (after removal of the superior cervical ganglion). A dilatation produced by epinephrin disappears more quickly if the adrenal blood is prevented from entering the circulation. In a previous paper (9) we say that this being so "there is every reason to expect that asphyxia, which even according to Kellaway causes some paradoxical dilatation in the absence of the adrenals, will increase the reactivity of the pupil to this ordinary output." Kellaway professes to find that the phrase "every reason to expect" "does not appear to have any definite

significance." We should have thought it self-evident that since asphyxia, as such, that is to say in the absence of the adrenal epinephrin, can cause dilatation of the sensitised pupil and since epinephrin can also cause dilatation there would be every reason to expect that a given output of epinephrin would cause a greater effect when favored or reinforced by the action of asphyxia than in the absence of asphyxia, and that accordingly the fact that a paradoxical reaction was more easily elicited or elicited in greater strength with intact adrenals than in their absence would not of itself show that asphyxia augments the rate of output of epinephrin.

We have observed in non-anesthetised cats (some days after transection of the cord in the cervical region) that the giving of ether increased the reactivity of the pupil, sensitised by previous removal of the superior cervical ganglion, to adrenal vein blood collected in a cava pocket, so that with a given time of collection a reaction was obtained where none had been obtained before, or a good reaction was elicited where only a small one had been got before the ether. At first sight this looks like a proof that ether augments the epinephrin output even after cervical cord section. And a good many statements with no definite basis exist in the literature to the effect that anesthetics cause an accelerated output. All that was necessary to dispose of this interpretation of the experiment mentioned, was to inject a definite dose of adrenalin before and after administration of ether. The pupillary response to one and the same dose was increased by the ether. The explanation we think is obviously that ether, which itself causes a dilatation of the sensitised pupil, favors the dilating action of the adrenalin, just as asphyxia does. Depression of the pupillo-constrictor activity, however produced, would bring about such an effect, and Langley (11) points out that paralysis of the ciliary ganglion by nicotine, for example, may complicate observations made by the aid of the pupillary paradoxical reaction. Kellaway's experiments on the excised iris throw no light upon the question.

His observation that when the aorta is clipped asphyxia causes dilatation of both pupils, whereas on releasing the aorta there is a preferential dilatation of the sensitised pupil simply shows that enough epinephrin secreted at the ordinary rate has been collected in the adrenal vessels and the cava to give a good or a maximal pupillary paradox when it is allowed to move on after release of the aorta. The experiment is only a repetition in a crude form of our own observations on the measurement of the epinephrin output by the pupil reaction, and the paradoxical reaction is also obtained without asphyxia.

It is a complete mistake to impute our negative result in asphyxia to the condition of the animals in consequence of the operation practised by us to obtain the adrenal vein blood. *The paradoxical pupillary reaction is excellently obtained at the time when we are inducing asphyxia after having prepared the cava pocket for collection of the adrenal blood and thereafter in the course of the experiment.* According to Kellaway this proves that the epinephrin output is augmented. We collect the

adrenal blood at this time. We estimate the concentration of epinephrin in the blood and, knowing the rate at which the blood was collected, we calculate the output of epinephrin per minute and we find that the output has not been changed by the asphyxia. The "clear and logical conclusion" is not that the phenomenon of the acceleration of adrenalin secretion supposed to be evidenced by the pupillary reaction fails under the conditions of our experiments, but that no sensible acceleration is produced by such grades of asphyxia as we have employed.

This ought to be sufficient to dispose of the suggestion that the operative procedure adopted to obtain the adrenal blood vitiates our results. But a few words on the development of the technique may prevent misunderstanding. Permanent ligation of the abdominal aorta just above the bifurcation, of the renal arteries and veins and of the inferior cava was not practised until it was shown that it did not cause any demonstrable effect on the epinephrin output. In one of our earlier papers (7) it is stated under "technique" that "where the eye reactions are used alone the cava pocket need not be permanent. For certain purposes the temporary closing off of the pocket for a minute or two at a time is all that is necessary, and in the interval the circulation proceeds practically in the normal way. A clamp is applied just above the iliac veins. The renal veins are then clamped and the segment of cava emptied of blood by gently stripping it upwards. Finally a clamp is put on the cava above the adrenal veins. Only a few seconds are occupied in the adjustment of these clamps. Small veins entering the cava segment have been previously tied." It was then tested whether permanent ligation of these vessels made any difference in the results. No difference having been found we thereafter tied these vessels as a matter of routine, and in our papers where it is simply stated that "the cava pocket was formed" it is implied that the vessels tied were only the abdominal aorta, cava, renal arteries and veins and the small veins. For blood pressure assays it was sometimes found advantageous to tie the superior mesenteric artery and the coeliac axis to eliminate irregularities in the curve. Before this was done numerous observations were made which failed to reveal that the epinephrin output was at all affected by this procedure. We have continued to tie these vessels or sometimes only the superior mesenteric, in some of our experiments, because a higher blood pressure and better blood flow through the adrenals and their nervous mechanism are thus insured. Their ligation is not in any way inherent in our method and in all our investigations there are plenty of experiments in which they were not tied, for example, the last experiment on asphyxia published by us (9). In our large series of experiments on strychnine, nicotine and other drugs the superior mesenteric artery and coeliac axis were not ligated We have also measured the epinephrin output in blood collected from one adrenal of a dog through a lumbar incision without opening the peritoneum and found no essential difference in the results from those of our other experiments.

SUMMARY

1. Our previous conclusion that the adrenal epinephrin output is not essentially concerned in the hyperglycemia induced by ether narcosis and asphyxia is confirmed. In rabbits which have survived the removal of both adrenals and have recovered from the operation and whose livers are well filled with glycogen, hyperglycemia is caused by these procedures just as in normal rabbits. We reaffirm our position that for these forms of experimental hyperglycemia and for the hyperglycemia caused by piqûre the so-called adrenalin hypothesis should be abandoned.

2. We do not find that any real evidence has been adduced by Kellaway that the epinephrin from the adrenals has a demonstrable share in the production of asphyxial hyperglycemia. His experiments really confirm our conclusion that interference with the epinephrin output does not modify essentially the hyperglycemia caused by asphyxia.

3. Kellaway has produced no evidence that asphyxia (or anoxemia) causes a demonstrable increase in the rate of epinephrin output from the adrenals. Despite his statement that "the results of the different experiments fully justify the conclusion that the paradoxical pupil reaction is a good index of the epinephrin output," we do not believe that by looking at the eye of an intact cat he can tell the amount of epinephrin coming off from the adrenals per unit of time before, during or after asphyxia, whereas we can obtain these amounts by collecting adrenal vein blood and assaying its content of epinephrin. The real reason for the difference in our conclusions as to the influence of asphyxia upon the rate of epinephrin output is that we have attacked a quantitative problem by direct quantitative methods instead of trusting to ambiguous reactions which are not even specific qualitative reactions for epinephrin. Our data, therefore, have a very different value for the determination of changes in the rate of epinephrin output.

4. Kellaway is completely mistaken in imputing our results on the epinephrin output in asphyxia to the condition of the animals in consequence of the operation practised by us to collect the adrenal vein blood. The paradoxical pupillary reaction is excellently obtained at the time when we are inducing asphyxia after having prepared the cava pocket for collection of the adrenal blood and thereafter in the course of the experiment. According to Kellaway this proves that the epinephrin output is augmented at the very time when we are collecting the blood. We ought therefore to be able to detect the augmentation

by our method. Since we do not detect an increased output by assaying the very blood in which the epinephrin is carried we are compelled to conclude that Kellaway's interpretation of the pupillary reaction as demonstrating an increased output of epinephrin in asphyxia is erroneous.

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THE EXPERIMENTAL PRODUCTION OF EDEMA AS RELATED TO PROTEIN DEFICIENCY

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INTRODUCTION

The shortage of food in general and of certain foodstuffs in particular, which occurred as one of the results of the recent war, has been the cause of a number of forms of malnutrition among which is that called "war edema" or "war dropsy." This is a form of malnutrition that has been recognized only recently, and the characteristics of the type of diet that has been the cause of its development have been a subject of considerable speculation and some research.

Budzynski and Chechowski (1) report a condition of dropsy among the inhabitants of Poland in 1915. The principal articles of food were potatoes (which were often of bad quality), soup and bread, with little fat and practically no milk or meat. Landa (2) reports a great number of cases of edema during recent war conditions in Mexico, 1915. The diet was made up largely of spinach and beets. Jurgens (3) in comparing conditions in various concentration camps of prisoners of war has studied the relation of an "edema disease" to the ordinary infectious diseases of prison camps and finds that the edema is not a part of the infectious syndrome as had been considered by some. He states that it results from a dietary deficiency and that where this was recognized and a more varied diet was given, the men soon recovered from the edema or its appearance was averted. He further states that often the full syndrome did not appear until the men were set to digging. Then 10 to 20 per cent of the men developed the edema disease. Wells (4), during his investigations in Roumania, 1917, observed a number of cases of dropsy among the people who were most seriously deprived of food. He describes this condition to the lack of fats, particularly the

lack of those fats containing the "fat-soluble A." He states: "We found, as far as we had time to observe it, that when the patients were given butter fat they were likely to get better. But, of course, when under treatment they always got milk, soup and other things." Park (5), who spent some time in war prisons, made a study of the prisoners who received war rations. He ascribes the condition to underfeeding, especially of fats and proteins, and to the large amounts of fluid ingested together with the increased amount of NaCl. Jaksche (6) describes an edematous condition due to the hunger conditions in Austria. He states that the edema develops when the calorie intake falls below 1400 calories per day. Knack and Neumann (7), Maase and Zondek (8) and Maase and Zondek (9) describe an edema mostly in men over forty. This edema they say is intermittent according to the diet. They state that this nutritive disorder is due to the combination of overfeeding of carbohydrates together with underfeeding of fat and an abnormally large intake of water from carbohydrates and soups. They further state that 100 grams of fat per day and a stay in bed is followed by improvement. Kraus (10) describes this condition of malnutrition occurring especially in the large cities and institutions of Germany. He states that the decrease in the quantity of food is undoubtedly the principal and perhaps the only cause of the increase in the death rate. The diet is described as a monotonous, coarse diet, not rich or tasty, with a lack of condiments and vitamines which led to a loss of appetite and this in turn to inanition. The intake of food was from 800 to 1300 calories per day where 15 per cent was indigestible carbohydrate, with very little fat and at the highest 50 grams of protein. This diet was used by men who were doing hard work. Guillermin and Guyot (11) report the same conditions in Poland and other war zones. McGarrison (12), describing the pathogenesis of deficiency disease, reports that inanition leads to a state of adrenal hypertrophy. He suggests that such a change may be held to account in great measure, through blood pressure changes, for the occurrence of "war edema." In an editorial (13) of the Journal of the American Medical Association on "what renders a diet inadequate," the question of the importance of the consideration of salts in the production and study of edema is discussed. Rubner (14), describing the food conditions and their effect on the race, describes war "edema" and refers to the lack of adequate protein in the diet more than any previous author. Beyerman (15) describes edema in the Netherlands where the symptoms disappeared promptly upon addition of fresh vegetables to the diet. Jansen (16) describes

the edema and suggests that the cause is probably an increase in the permeability of the capillary endothelium which alters the exchange of fluids and salts between the tissues and the blood. Vandervelde and Contineau (17) describe an edema of the lower limbs of patients who were deported from Germany. He states that there was a lack of food, deplorable hygienic conditions, and that there were disturbances in the cardio-vascular apparatus, accompanied by anemia and that dyspnea was brought on with little exertion. Therapeutic measures were dietary improvements and rest which were followed by the disappearance of all symptoms. Romano Tonin (18) reports a hunger edema in which polyuria is a constant accompaniment. Reach (19) observed that many subjects with edema had atrophic testes or cryptorchism. He states that in twenty-six cases of war edema where dietary changes were of no benefit, testicular apotherapy was tried and in fourteen cases the patients were cured. Denton and Kohman (20), while working on the dietary qualities of carrots, found that dropsy occurred in a large percentage of rats fed on a carrot diet, when the proportion of nitrogen had been reduced by the addition of some non-nitrogenous foodstuffs, such as fat and starch. In a preliminary note (21) of the present piece of work the writer describes the cure of rats which had developed edema on the low-protein-carrot-diet, by the substitution of 18 per cent pure casein for 18 per cent corn starch in the diet, all other factors remaining the same.

METHODS

Preparation of materials

Dried carrots. The carrots that were used in the dry diets were washed, trimmed and ground in an ordinary meat grinder, spread on glass trays which were supported in a drying oven through which air was passed which was heated to 37° to 40°C. The carrots were then stored until they were used in the diet, but were not left to stand more than one or two months.

Wet carrots. Carrots used in the wet diets were prepared as for drying but were used fresh in the diets.

Butter fat. Good quality of fresh butter was heated from 90° to 95°C. in order to separate the proteins and water from the butter fat which was decanted off.

Lard. Leaf lard was obtained from the market and rendered.

Casein. Skim milk was purchased in six-gallon lots. This was diluted with an equal volume of distilled water. The casein was precipitated with hydrochloric acid (250 cc. to 10 liters of H_2O) and filtered through cheesecloth. It was then ground with mortar and pestle and divided into six lots and each lot covered with 5 to 6 liters of distilled water slightly acidulated with acetic acid, and was left to extract for about 24 hours. The water was then filtered off through cheesecloth and the casein was ground in a mortar with pestle, diluted to 4 liters with distilled water and dissolved with sodium hydroxide (250 cc. of 50 per cent NaOH to 10 liters H_2O). When dissolved the solution was diluted to 6 liters or more and the casein was again precipitated, this time with glacial acetic acid. The water was filtered off through cheesecloth. The casein was again dissolved, as above described, and filtered through filter paper with the aid of a suction filter. The solution was again diluted and the casein precipitated with hydrochloric acid. The casein was then filtered out and washed with distilled water to remove the acid. The water was removed with the suction filter. The casein was then ground in 95 per cent alcohol, covered with 3 liters of alcohol and left to extract 48 hours. This extraction was repeated three times with 95 per cent alcohol and twice with ether, filtering the alcohol and ether off each time with the aid of the suction filter and mixing well the casein each time with the fresh extraction solution. The casein was then dried in air and stored.

This careful method for the preparation of casein with repeated extractions with alcohol and ether was used in order to be sure that the casein contained neither of the vitamines, "fat-soluble A" or "water-soluble B." In some previous work commercial casein was used after extended washings with dilute acetic acid and extractions with alcohol and ether. Animals fed on a diet in which this casein was used showed a retarded growth but none of the other symptoms commonly attributed to the absence of the fat-soluble vitamines.

Starch. Kingsford's cornstarch was used throughout the experiments.

"Water-soluble B" was extracted from wheat germ by two extractions, using 25 grams of wheat germ and 250 cc. of 95 per cent alcohol for each extraction. The alcohol containing the "water-soluble B" was mixed and dried with the other ingredients of the diets that were fed dry, and was dried on the proper amount of cornstarch which was used in the diets that were fed wet.

Salts

Salt II	grams
Sodium chloride	0.50
Ca lactate	1.57
Ca $H_4(PO_4)_2$	0.60

Salt VI	
Sodium chloride	0.50
Ca lactate	1.5507
Ca $H_4(PO_4)_2$	0.6232

Salt III	grams
Sodium chloride	0.1739
Sodium sulphate	0.318
Sodium H_2PO_4	0.347
Potassium H_2PO_4	0.954
Ca $H_4(PO_4)_2$	0.540
Ca lactate	1.300
Fe lactate	0.118

Salt III is essentially the salt which was shown by McCollum (22) to supply all the necessary mineral for normal growth in rats when the diet was made up of purified food substances. Instead of $MgSO_4$, as used by him, Na_2SO_4 was used to supply the same amount of sulphur. The change was made to reduce the amount of magnesium as carrots have a high magnesium content. Salt II is used to supply the minerals that are deficient in carrots. The two salt mixtures were used in such proportions as to make the salt content of the diet as nearly as possible the same as that of milk.

Making up diets and feeding rats

Wet diets. The cornstarch with the extract of wheat germ, butter fat and salt was weighed, distilled water was added and the mixture boiled to a clear stiff paste, while stirring constantly. The starch was boiled only a minute or two in order to break up the starch cells and make them more easily digested. To this paste was added the proper weight of fresh carrots, ground in an ordinary meat grinder. This mixture was made up every two days and kept in the ice box between feedings. Fifty grams of this wet food mixture were weighed out, recorded and given to each rat each day and the amount scattered and left in the dish was estimated on the next day and recorded. The amount left was not weighed because considerable moisture would evaporate and this made weighing quite as subject to error as estimation. The difference of the two weights was used as the food consumption of the rat for that day. As some of the animals scattered their food a great deal, considerable more evaporation took place in the case of the food of some animals than of that of others. Although this evaporation was considered in estimating back what the rat had not eaten, there was, no doubt, some unavoidable error in the estimation of the food consumption of the rats on this diet. Still it was a

method, with some degree of accuracy, to compare the relative amount of food consumed and a means by which one would detect any great variation in the food consumption of an animal, or any great difference among different animals. Knowing the calorie value of the ingredients which make up a diet, and the weight of the completed diet, the calorie value per gram of diet was determined by dividing the total calorie value by the total number of grams in the mixture. From this figure and the food consumption record of each rat the daily calorie intake of each rat was determined.

Dry diets. The cornstarch, butter fat and salts were weighed out, distilled water was added and the mixture boiled to a stiff paste. To this the alcoholic extract of wheat germ was added and thoroughly mixed. The proper weight of previously dried carrots was then added and the entire mass well mixed, making a very stiff paste. This was then divided into equal portions by means of two tubes, one about 4 inches long and about 1 inch in diameter, and the other just enough smaller to fit into the larger one closely. The larger tube was filled with the stiff paste. This was pushed out by means of the smaller tube which had one end closed by means of a close-fitting cork. The column of paste was then divided into four equal parts with a knife. These cakes of paste were put on a glass tray and dried in the oven described above and stored for use. Knowing the value in calories and the dry weight of the bulk of food made up, the value of each small cake, in calories and grams, was determined by dividing the value of the entire quantity by the number of cakes into which the mass was divided. A record was kept of the number of cakes eaten by each rat, and from this record the food consumption of each rat for each week of the duration of the experiment was determined.

Care of the animals

The animals used for the experiments were raised in this laboratory from stock rats kept for that purpose. These rats were fed a variety of foods—milk, mixed grain, fresh carrots, bread and occasionally meat. The animals were weighed and their cages changed and sterilized once each week.

The animals used for experimentation were taken at from about 50 to 60 grams and only when they had shown normal growth previous to being put on the experimental diet. Each experimental animal was kept in a separate cage and a record was kept of his food consumption.

The cages used were made entirely of metal. The trays were covered with paper. The cages were changed and sterilized by live steam once a week for the rats eating dry diets, and twice a week or more, as thought necessary, for those rats kept on a wet diet. The animals on the dry diet were supplied with distilled water. Those on the wet experimental diet were given no water as the water content of the food was quite high.

RESULTS

General description of animals on a low protein diet. When normal growing rats are put on the low-protein carrot diet, they stop growing at once and very soon show a gradual loss in body weight, more rapid for some animals than for others. They may lose as much as 30 to 33 per cent of their original weight in nine or ten weeks and 46 per cent in sixteen weeks. Along with this loss in weight there is a decrease in activity, not marked at first but in later periods of the experiment very decided. The animal sits quietly in the cage most of the time with its back humped up, its eyes only partly open, and head bent down between its fore legs. It is usually asleep except while eating. When moving about it seems dull, moves slowly, the more so as the experiment progresses.

The coat soon becomes rough. The hair becomes fuzzy and dirty looking as compared to that of the normal rat. If the animal lives for a long time on this diet the hair comes out in patches until sometimes almost the entire body is bare. The skin becomes dry and scaly and the animal is very subject to lesions on the tail, ears and nose. The lesions are scaly red patches on the exterior surface of these parts, are never wet and do not contain pus but are slightly elevated.

There is a great decrease in general resistance. While the stock rats and rats on an adequate diet have never been troubled with lice, it is very difficult to keep an animal on this diet free from lice. Any exposure to cold also usually leads to death while the normal animal does not suffer from the same exposure. The animal shows a great muscular weakness with subsequent wasting. Anemia is indicated by the pallor of the ears as contrasted with the pink ears of a normal animal. One factor of this anemia seems to be a decrease in blood volume, for it is more difficult to draw blood from such an animal than from the normal animal.

The duration of life of rats on the low-protein-carrot-diet varies among different rats from a very short time (2 weeks) to 3 months and

in some cases more. Toward the end of this time there is usually extreme weakness and loss of appetite. At this stage quite a number of the animals die without showing any edema. In a larger percentage of cases, however, an edema develops, this percentage in the rats on the wet diet being higher (86 per cent) than in the rats on the dry diet (55 per cent). (See table 3). This edema is usually detected first about the face, especially about the eyes and cheeks. This may be noticed in some cases several weeks before any extreme edema occurs. A more extreme type is an edema about the chest and fore legs. This can first be detected by a thickening of the skin. The animal may die in this stage, or the edema may disappear and return intermittently until death, or a very extreme edema may develop where as much as 10 cc., or occasionally more, of fluid, either gelatinous or liquid, may collect under the skin of the chest in the form of a large "blister." Even this extreme type of edema may often disappear and return intermittently on this diet, but a gain in body weight has never been noticed in this partial recovery; on the contrary, there is a decided weakening from one time to the next which finally leads to death unless the diet is corrected soon enough. In some animals the edema is in the form of a general anasarca, a fluid being collected in the serous cavities, both pleural and peritoneal. Except in extreme cases this could not be detected with certainty until after the death of the animal when the cavities were opened and the fluid pipetted out and measured. The amount of fluid varies from 0.5 to 2 or 3 or occasionally more cubic centimeters. Sometimes there is an abnormal amount of fluid in both cavities and sometimes in either one or the other.

Post-mortem examinations were made of all the animals that died as a result of being fed the low-protein-carrot-diet. In all cases there was a depletion of fat in the body. A very common finding was pneumonic lungs. There was a marked decrease in size of the testicles in the male, which was easily detected in the living animal. There was usually a congestion of the lymph glands especially those in the neck.

EXPLANATION AND DISCUSSION OF TABLES AND RESULTS

The edema is not due to a deficiency of fats or fat-soluble vitamine. Wet diets. The lack of fat and "fat-soluble-vitamine A" has been emphasized as much or more than any other factor as the cause of edema in the regions which were most seriously deprived of food as a result of

the recent war. This idea, no doubt, developed from the fact that there was a great shortage of fats and dairy products in these regions, and because it has been previously shown that xerophthalmia, a dryness of the conjunctiva, which eventually leads to blindness, and an edema of the eyelids, occurs in rats as a result of being fed a diet entirely free from or low in its content of the fat-soluble vitamine as reported by Mendel (23), McCollum (24), Steenbock (25), Funk (26) and others. Edema as it has been produced in rats in this laboratory, however, is much more extensive and entirely distinct from that which occurs as reported in xerophthalmia for it may occur subcutaneously, covering large areas or almost the entire surface of the body of the rat, and may take the form of a general anasarca, with large amounts of fluid (5 to 6 per cent of the body weight) collected in the serous cavities.

My experimental results apparently prove that this edema as produced in rats in this laboratory is not a result of the lack of the fats or the fat-soluble-vitamine. The diet used in this work to produce edema in rats was made up largely of carrots, with additions of starch, fats and salts, carrots being the only source of protein. This diet was chosen because it was with such a diet that this edema was first produced in rats while studying the nutritive value of carrots (20).

Table 1 shows the results of feeding four groups of rats on diets in which the carrots and salt content was the same throughout and the caloric value was practically the same. The quantity of fat and starch was varied. The amount of each constituent of the diet is shown in grams and calories. In these diets fresh carrots were used, of which the calorie value is about 0.4 calorie per gram of fresh carrots. In diet I (X butter), 60 grams of butter fat were used, this supplying 1 gram of butter fat for every 63 calories of the diet. McCollum and Davis (22) have shown that 5 per cent butter fat in a diet of purified foodstuffs was sufficient for normal growth in young rats. This amount of butter fat used by them supplied 1 gram of butter fat for every 88 calories in the diet. The amount of fat supplied in X butter diet should be sufficient for rats.

In diet II (X lard) the fat was supplied in the form of lard, the same amount being used as was supplied as butter in the first diet. In diet III (no fat) no fat was supplied but the quantity of starch was increased. In diet IV (K + A − B) the butter was increased to twice the amount that was used in diet I (X butter). The calorie value was kept practically the same as in diets I and II by increasing the amount of starch where the fat was decreased. This increase of butter was made to

GENERAL EXPLANATION OF TABLES

The symbol "K" wherever it occurs in the tables denotes 550 grams (dry weight) of carrots, 228 grams of starch and 120 grams of fat.

"x" butter or "x" lard denote diets in which there are 60 grams of butter or lard respectively.

"A" represents fat-soluble-A in 120 grams of butter and "B" denotes the alcoholic extract of 50 grams of wheat germ. The presence or absence of these vitamines in the respective diets is indicated by the presence of a + or − sign before the letter. For example, K + A − B means that the standard diet K (550 grams of dried carrots, 228 grams of starch, 120 grams of fat) contained fat-soluble-A but was deficient in water-soluble-B.

TABLE 1

Table of wet diets with results of feeding

	I × BUTTER		II × LARD		III NO FAT		IV K + A − B	
	Grams	Calories	Grams	Calories	Grams	Calories	Grams	Calories
Carrots	4500	1800	4500	1800	4500	1800	4500	1800
Starch	360	1440	360	1440	456	1824	228	912
	BUTTER		LARD				BUTTER	
Fat	60	540	60	540	0	0	120	1080
Salt II	19.2	0	19.2	0	19.2	0	19.2	0
Salt III	16.4	0	16.4	0	16.4	0	16.4	0
Total		3780		3780		3624		3792
Number of rats on diet	7		6		5		3	
Number of rats developing edema	3		3		2		3 only slight	
Per cent of rats developing edema	42⅞		50		40		100	

Table 1 shows the results of a study of the effect of certain fats and the fat-soluble vitamine on the production of edema in rats. Edema occurs in rats on all four of the diets so butter fat or the fat-soluble vitamine in butter does not prevent the edema, neither does it decrease the percentage of edema. Even when diet IV was fed with the butter fat content of 120 grams, all of these rats developed edema The table also shows that fat is not the cause of the edema, for it occurred with and without fat in the diet.

insure sufficient intake of butter fat and fat-soluble-vitamine by the rats as their food consumption seemed to be somewhat less than that of the normal rat and it was thought that possibly not enough of the fat was obtained in the smaller food consumption.

The lower part of table 1 shows the number of rats fed each diet, together with the number and percentage of them that developed edema. It is of interest to note that where the greatest amount of butter fat was used the greatest percentage of rats developed edema and that, although there is little difference, the lowest percentage of cases of edema developed on the diet in which there was no fat supplied. This shows that fat and fat-soluble-vitamine do not prevent edema from developing in rats if the protein content of the diet is very low.

The edema is not due to a deficiency of water-soluble vitamine or salts. Dry diets. Table 2 shows the results of a series of experiments in which dried carrots were used in making up the diets. The water used in making up the diet was also evaporated so the diet was administered in a dry form. The carrots dried to practically 11.8 per cent of the wet weight, making the calorie value of the dried carrots 3.33 per gram of dried carrots. The same constituents were used as those used in the diets given in table 1 with the addition of an alcoholic extract of wheat germ in some of the diets. Although carrots have a considerable amount of the water-soluble vitamine this addition of alcoholic extract was made to insure a sufficient intake of this vitamine, for the lack of this vitamine is known to cause beri-beri which is often accompanied by more or less edema (28).

Diets V (K + A + B) and VI (K + A − B) each contain the large amount of butter fat used in diet IV of table 1. In addition diet V (K + A + B) contains the alcoholic extract of 50 grams of wheat germ which supplied the extract of 1 gram of wheat germ for every 76 calories of the diet. McCollum (29) has shown that 3 per cent of wheat germ supplies sufficient water-soluble-vitamine for almost normal growth in young rats. This amount supplies one gram of wheat germ for every 133 calories in the diet. The amount of wheat germ used in the diets shown in table 2, together with the water-soluble-vitamine in carrots should supply enough of the water-soluble-vitamine to eliminate the lack of this vitamine as the cause of the edema which develops in these rats. This evidence is strengthened by comparing the percentages of edema on diets IX and X where 60 grams of butter fat are supplied in each but the water-soluble-vitamine is supplied only in X from which diet $33\frac{1}{3}$ per cent of the rats developed edema whereas only $16\frac{2}{3}$ per cent

TABLE 2

Table of dry diets with results of feeding

	V K+A+B		VI K+A−B		VII K−A+B		VIII K−FAT+B		IX ×BUTTER		X ×BUTTER+B		XI ×LARD		XII K+A+B+2×SALT	
	Grams	Calories	Grams	Calories	Grams	Calories	Grams	Calories	Grams	Calories	Grams	Calories	Grams	Calories	Grams	Calories
Carrots (dry)	550	1815	550	1815	550	1815	550	1815	550	1815	550	1815	550	1815	550	1815
Starch	228	912	228	912	228	912	490	1960	360	1440	360	1440	360	1440	228	912
	Butter		Butter		Lard				Butter		Butter		Lard		Butter	
Fat	120	1080	120	1080	120	1080	0	0	60	540	60	540	60	540	120	1080
Salt II	19.2	0	19.2	0	19.2	0	19.2	0	19.2	0	19.2	0	19.2	0	38.4	0
Salt III	16.4	0	16.4	0	16.4	0	16.4	0	16.4	0	16.4	0	16.4	0	32.8	0
Extract of wheat germ	50	0	0	0	50	0	50	0	0	0	50	0	0	0	50	0
Total		3807		3807		3807		3775		3795		3795		3795		3807
Number of rats on diet	6		6		1		2		6		3		2		12	
Number of rats developing edema	3		2		0		0		1		1		0		2	
Per cent of rats developing edema	50		33⅓		0		0		16⅔		33⅓		0		16⅔	

Table 2 shows the results of a further study of edema in rats as produced by inadequate diets. The results given here are produced by feeding dry diets and supplying the rats with an unlimited amount of distilled water. In addition to a variation in the fats in these diets, the salts and the extract of wheat germ were varied. As in table 1, this table also shows that butter and the fat-soluble vitamine in butter do not alter the occurrence of edema in rats when there is a deficiency in protein. This table also shows that an increase in the salt content or the addition of the alcoholic extract of wheat germ does not influence or prevent the occurrence of edema.

developed edema on diet IX with no water-soluble-vitamine supplied. The percentage of rats developing edema as a result of the diets with lard or no source of fat, diets XII, VIII and XI, is again low but we cannot interpret this fact as meaning that fat is a cause of the edema, for in table 1 on diet III (no fat) 40 per cent of the rats developed edema. So the presence of "water-soluble B.' does not prevent edema from developing in rats on a low protein diet. McClugage and Mendel (30) report very poor utilization of salts where a diet is high in its content of indigestible cellulose from vegetables. In order to insure a greater assimilation of salts from the diet of carrots, which is very high in its content of indigestible matter, a diet was prepared in which the quantity of salt was doubled, diet XII (K + A + B + 2x salt) the same as diet V (K + A + B) except for its salt content. The percentage of edema was not markedly altered by the higher salt content in the diet. As the retention of salts by the tissues is a very well-known cause of edema, it is of interest to note that the percentage of edema developing on this diet of double salt content is not increased.

How does the water content of the diet influence the edema? Another factor in the production of edema is the water intake. The total percentage of edema developing from the diets of table 1 (wet diets) which were about 84 per cent water, is 52.4 per cent. The total percentage of edema developing from the diets of table 2 (dry diet) is 23.7 per cent. As the two series of experiments were conducted at different times the rats could not be selected from the same litters. In order to study this factor more carefully, another series of experiments was conducted, the results of which are recorded in table 3. In addition to this factor the salts were again varied to control this factor more completely, and a control diet was used in which casein was added as a source of protein.

In table 3 diets V (a) and XII (a) (dry diets) are the same as diets V and XII, table 2, respectively. Diets XIII and XIV (wet diets) are the same as V (a) and XII (a) table 3 respectively except for the water content. Eighty-six per cent of the rats on the wet diets developed edema whereas only 55 per cent of the rats on the dry diets developed edema. This percentage is much higher than that of the earlier work shown in tables 1 and 2. This is explained by the fact that the course of the edema was not so well understood in the early work. This edema is intermittent and may come on in a short time and disappear in a day. If no special notice was made of that particular animal at the time of the edema the case was overlooked. In the later work the animals were

watched more closely, hence no cases of edema were overlooked which accounts for the higher percentage of edema in the later experiments. In addition to this difference there is a decided difference in the length of time before the edema develops on the dry and wet diets. Rats on the wet diets develop edema and die in from 9 to 13 weeks, most of

TABLE 3

Table of dry and wet diets with results of feeding

	DRY				WET				DRY	
	V (a) K+A+B		XII (a) K+A+B +2×Salt		XIII K+A+B		XIV K+A+B +2×Salt		Control	
	Grams	Calories	Grams	Calories	Grams	Calories	Grams	Calories	Grams	Calories
Carrots	550	1815	550	1815	4500	1800	4500	1800	550	1815
Starch	228	912	228	912	228	912	228	912	84	336
	Butter		Butter		Butter		Butter		Butter	
Fat	120	1080	120	1080	120	1080	120	1080	120	1080
Salt II	19.2	0	38.4	0	19.2	0	38.4	0	38.4	0
Salt III	16.4	0	32.8	0	16.4	0	32.8	0	32.8	0
Extract of wheat germ	50	0	50	0	50	0	50	0	50	0
Casein	0	0	0	0	0	0	0	0	144	576
Total		3807		3807		3792		3792		3807
Number of rats on diet	11		9		7		8		3	
Number of rats developing edema	5		6		7		6		0	
Per cent of rats developing edema	$45\frac{5}{11}$		$66\frac{2}{3}$		100		75		0	
	55				86					

Table 3 shows several very interesting and conclusive results. First, when the diet contains a high water content the percentage of edema in rats which are fed the low-protein diet, is markedly increased, an increase from 55 per cent to 86 per cent. Second, a variation in the quantity of salts in the diet had no effect on the occurrence or percentage of edema. With the single salt portion 12 rats out of 18 developed edema. With the double salt portion 12 rats out of 17 developed edema. Third, and very important, the table shows that when a sufficient amount of an adequate protein replaces an amount of cornstarch, of equal caloric value, no edema developed in rats as a result of feeding this carrot diet. Furthermore, on examination of figure 2, it will be noted that rats fed this diet, (the same as the low-protein-carrot-diet except for the protein content), grow and reproduce normally.

them being dead at the end of the 11th week. Quite a large number of the rats on the dry diets live 18 or even 30 weeks. However, a small number die as early as the 9th week. So an increased water intake increases the number of cases of edema developing in rats on a low-protein-diet.

In case of food shortages, therefore, when it is necessary to use only limited amounts of protein or to use proteins of a poor quality, it is of practical importance to use these foods in a relatively dry form rather than in the form of soups as is usually done to make the food more "filling."

The salt content of the diets in table 3 did not change the percentage of cases of edema. Out of seventeen rats on the double salt diet, twelve developed edema. Out of eighteen rats on the lower salt diet, twelve rats developed edema.

The edema is due to protein deficiency. In the control diet 144 grams of casein replaced 144 grams of starch, the caloric value, vitamine content, fat and salts remained the same. It should be noted that there were no cases of edema developing on this diet. Furthermore, the growth curves were normal (see fig. 1) for the rats on this diet. The females produced normally but showed some difficulty in rearing their young. However, rat 304 (see growth curve, fig. 1) gave birth to eight young weighing 35 grams at birth. After twelve days they had not shown normal growth and well-being. At this time the litter was reduced to four. At the age of six weeks the average weight of the four young was 55 grams and at the age of eight weeks, when they themselves ate of the control diet, the average weight of the four rats was 92 grams. They were active and in general good condition. This shows that the low-protein-carrot-diet used throughout the work to produce edema was adequate except for its protein content, for when sufficient adequate protein was added, no edema occurred and the rats grew and reproduced normally.

This control diet with adequate protein supplied by the addition of casein to the low-protein-carrot-diet was also used as a cure for rats that had developed edema on the low-protein-carrot-diet. In a number of cases a cure of the edema resulted when the edematous rat was put on this diet with adequate protein content, and the rat in question would become more active, would show a rapid increase in weight and also soon increase his food consumption. As long as the rat remained on this diet the edema did not return, but as soon as it was put back on the low-protein-carrot-diet it would lose weight, general symptoms

of malnutrition would appear and after a time the animal would die, some of the rats developing edema the second time. The most interesting and striking case is that of rat 165 whose weight curve is shown in figure 1.

Rat 165 was put on diet V (K + A + B), table 2, and after eating this diet for 11½ weeks he had a large "blister" on the chest between the forelegs and showed a marked subcutaneous edema of the left

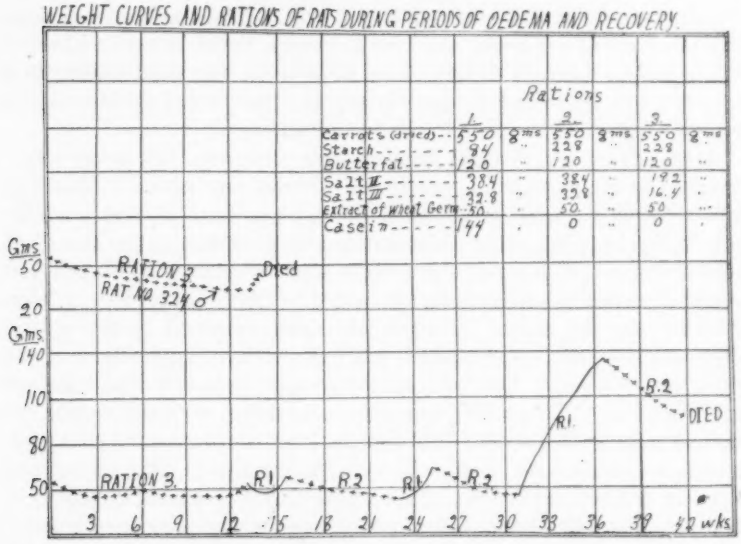

Fig. 1. The curve of rat 324 is a typical weight curve of rats kept on the low-protein-carrot-diet. There is a gradual loss in weight but when edema sets in a gain in weight occurs which is due to the accumulation of fluids in the tissues. No attempt was made to cure this rat.

The curve of rat 165 shows a gradual loss of weight while it was kept on the low-protein-carrot-diet, with an increase at the end of the 16th week due to the accumulation of fluid. Recovery followed a change in diet, the change consisting in giving adequate protein, with not much gain in weight, however. This animal developed a marked edema three times and was cured each time by a change to a diet containing adequate protein, a gain in weight and marked improvement in general condition resulting. During the last period of protein feeding, which was a relatively long period, the rat grew normally, its hair grew out perfectly over its entire body and it was very active. The rat finally died while on the low-protein-carrot-diet, without developing edema. At autopsy the lungs were found to be pneumonic, which was probably the cause of death.

side of the neck. After 3 days this edema had disappeared but after a few days it returned, the weight increasing from 45 grams to 52 grams due to the accumulation of fluid subcutaneously and in the tissues. At this time, 8 days after edema appeared for the first time, the rat was changed to the control diet (see table 3). In 2 days the edema about the chest and neck was entirely gone and only slight edema of the eyes remained, which cleared up in 2 more days. The weight of the rat had gone down to 47 grams. This finding is explained by the well-known observation of Bischoff and Voit (31) who noticed a decided loss in weight of a dog who had been kept on a diet of bread for 41 days, losing weight during this time; then when meat was administered in a quantity enough to cause a protein deposit, there was a decided loss in weight on the first day due to the loss of water.

After the above rat had been on this adequate diet a few days, increase in body weight followed but without any edema. This diet was continued for 2½ weeks when the rat was put back on the low-protein-carrot-diet but with the double salt portion. At the end of 6½ weeks the animal showed some slight signs of edema. This left but returned much more pronounced in a few days, again with a large "blister" on the chest. The rat was again returned to the control diet with adequate protein which was followed by disappearance of the edema in 14 hours. Again the animal became more active and showed rapid growth. His general appearance, however, was not normal for he had a great loss of hair and was not kept on the control diet with adequate protein long enough for new hair to grow. The animal was kept on this diet for 2 weeks during which time he gained 22 grams in body weight. He was again put back on the low-protein-carrot-diet with the double salt content. This was followed by the characteristic loss in weight and activity. During this period there was some growth of hair in the bare patches but the hair was not like the hair on the normal rat, it being very soft and fuzzy. After being on this diet for 5½ weeks the rat developed edema for the third time, this time about the neck and face but without the large "blister." The control diet was given, and normal growth was resumed as shown in the weight curve in figure 1. In 2 weeks there was rapid growth of hair which was firm and normal. There were some patches of the fuzzy dirty hair left which resulted from the poorer diet, but this hair was gradually replaced by normal hair. The rat was kept on this diet for 6 weeks and at the end of this time was in splendid condition, active, had fine coat of hair and eyes in excellent condition. The rat was put back on

the low-protein-carrot-diet and lost weight again, this time more rapidly. At the end of about 4 weeks the animal died without showing any signs of edema. He had been quite weak for some time and seemed to breathe with difficulty. After death the lymph glands were slightly congested, the lungs were congested and pneumonic. The cause of death was probably pneumonia which was contracted due to his low resistance as a result of the diet deficient in protein.

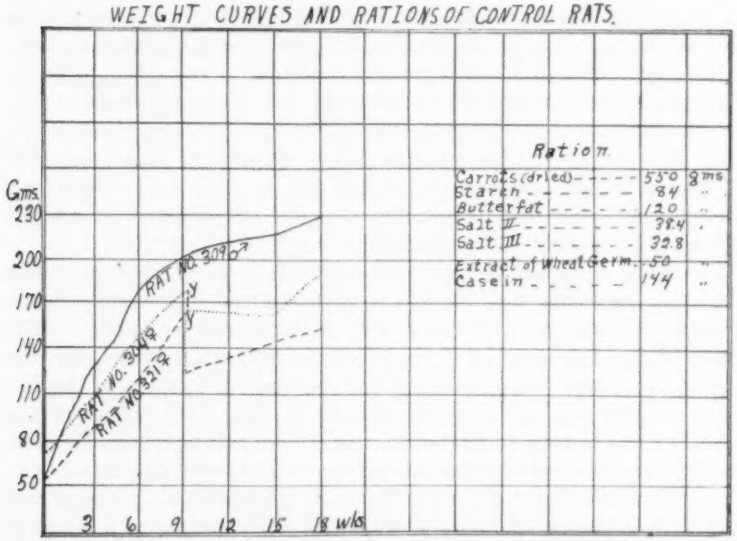

Fig. 2. The weight curves of the three control rats are given in this chart. These rats were fed the carrot diet containing an adequate amount of protein. (One is a male, the other two are females.) All showed normal growth and reproduction. The females gave birth to normal young (at *y*), but had some difficulty in rearing them. However, rat 304 succeeded in rearing a litter of young to the weaning stage and then the young ate the control diet and also grew normally with the control diet as their only source of food. These charts show that rats kept on a carrot diet with adequate protein do not develop edema.

Cure did not result in all cases of edema where the control diet was given to the rat. In a number of cases the rats were so feeble they refused to eat the food. The rats were usually left on the low-protein-carrot-diet until the edema had developed to an extreme degree and probably to such a degree that death would come before a sufficient

quantity of protein was assimilated. However, the writer feels confident that recovery would result in all cases if the diet were corrected soon enough, for in the control rats not only was edema averted, but none of the symptoms appeared that precede the edema where it develops.

In addition to the feeding done with the low-protein-carrot-diet, a lot of 3 rats was fed on a diet made up of 87 grams cornstarch, 5 grams butter fat, 4 grams of a complete salt mixture, 2 grams agar, 2 grams of casein and the alcoholic extract of 6 grams of wheat germ. This diet is adequate except for its protein content which makes up only 2 per cent of the diet. The general condition of the rats of this lot throughout the course of this experiment was about the same as that of the rats on the low-protein-carrot-diet, losing weight at about the same rate and having the same condition of hair and skin. One out of three of these rats developed edema just as those rats on the low-protein-carrot-diet did. The other two did not develop edema but showed the same signs of malnutrition.

The edema is not due to the low-calorie intake of the edema rats. Some observers have said that the edema that occurred in the war-stricken countries during the war was due to a low intake of calories. The food consumption records of my experimental animals show that the rats on the low-protein-carrot-diet did not consume as much of the diet as those who received adequate protein in their diets, although the same amount of food was given to each. Low calorie intake therefore had to be considered as a possible cause of the edema in these rats.

That low calorie intake is not the cause of the edema in the rats in this work is shown by figure 3, which gives the results of a study of this factor in the production of edema. Curve *I* is a composite weight curve of the three control rats which received 18 per cent of purified casein in their diet. Curve *III* shows the average weekly food consumption of these rats in calories. Curve *II* is a composite weight curve of six rats on the low-protein-carrot-diet. Curve *IV* shows the average weekly food consumption of these rats in calories. The only source of protein in the diet of the rats of curve *II* is the carrot in the diet. Curve *V* is a composite weight curve of five rats whose food consumption was limited in calories to that amount shown in curve *IV*. The diet of these rats was made up the same as that of the rats whose weight curve is represented by curve *II*, but with sufficient casein replacing starch in the diet to give each rat 1 gram of casein per day, which is approximately the amount of casein consumed by the control

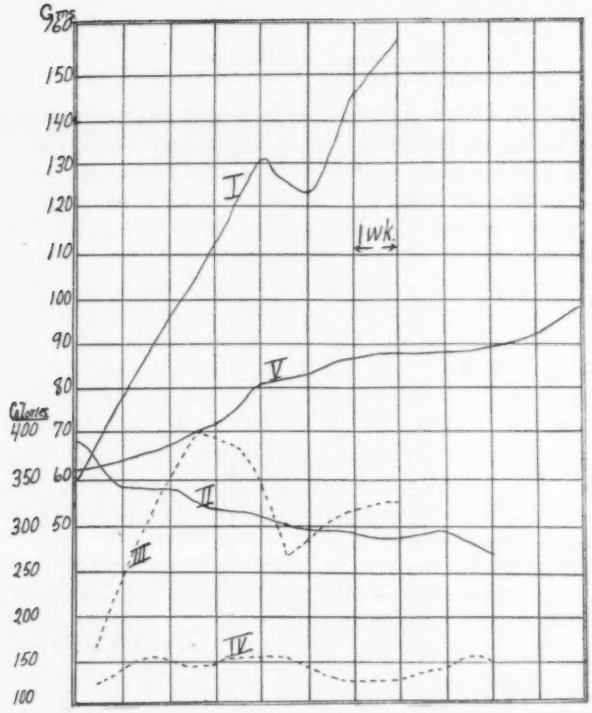

Fig. 3. The curves of figure 3 show that the low calorie intake of the rats used in this work is not responsible for the development of the edema.

Curve *I* is the composite growth curve of rats with 18 per cent casein in their diet. Curve *III* represents their average weekly food consumption in calories. Curve *II* is the composite weight curve of rats on the low-protein-carrot-diet. Curve *IV* represents their average weekly food consumption in calories. Curve *V* is the composite growth curve of rats whose food consumption is limited to that represented in curve *IV*, i.e., the same as the rats whose weight curve is shown by curve *II*, which means that the rats of curve *V* received a low-calorie diet; but the rats of curve *V* were fed adequate protein, 1 gram of casein per day being fed and cornstarch, of the caloric value of the gram of protein added, being withdrawn. So the rats in curve *V* received a low-calorie diet with adequate protein. These rats (curve *V*) did not show edema but slowly gained in weight. As pointed out before, the rats of curve *IV* showed a large percentage of edema. Which proves that the comparatively low-calorie intake of the edematous rats was not the cause of the edema, for edema did not develop in the rats (curve *V*) receiving adequate protein with low-calorie intake.

I. Composite weight curve of 3 rats on carrot + 18 per cent casein diet.

II. Composite weight curve of 6 rats on carrot—low-protein diet.

III. Curve showing average weekly food consumption in calories of rats of curve I.

IV. Curve showing average weekly food consumption in calories of rats of curve II.

V. Composite weight curve of 6 rats on low calorie diet with adequate protein.

rats. There is a slow gradual gain in weight of these rats which was still continuing at the time the experiment was stopped. The rats showed a slight tendency to the lesions described in the rats kept on the low-protein-carrot-diet, which shows that these lesions are not specific for the absence of adequate protein in the diet, but due to an infection as a result of lowered resistance. These lesions could be cured by the application of vaseline. With the exception of the lowered resistance and the retarded growth, these animals appeared to be in excellent condition, the hair and eyes being normal as well as appetite and activity. If the low calorie intake was the cause of the edema in rats, these rats should have at least shown some edema at the time the experiment was stopped, for they had been kept on this low protein diet 14 weeks. The diet was fed in the wet form. When rats of the same age and weight had been fed the wet low-protein-carrot-diet (results shown in table 3) the rats were all dead at the end of 13 weeks and a large percentage of them had developed edema.

In additon to the work recorded in figure 3, some rats were fed a diet of even lower calorie value, the food consumption being reduced by 16 per cent, but each rat was still receiving 1 gram of purified casein per day. Some of these rats lost weight and died without showing edema and some were growing and in splendid condition when the experiment was stopped. So the low-calorie intake of the edematous rats is not the cause of the edema.

The edema is not due to some "toxic" effect of the carrots. The writer has been told that the people of Germany who used vegetables as such a large part of their diet and suffered from war edema are said to have called the disease "Rübenkrankheit." The low-protein-carrot-diets which were the cause of the development of edema in these rats had a very high carrot content and some might say that there may be some toxic product in the carrots that impairs the kidney or some other tissue of the body and makes it impossible to eliminate fluids from the body. This cannot be the case, for the control rats receive the same proportion of carrots in their diets and are perfectly normal. Furthermore one lot of three rats was fed on a low-protein-diet (2 per cent casein) with no carrots, but with the calories, usually supplied by carrots, supplied by cornstarch. The rats followed the same general course and one developed edema just as the rats on the low-protein-carrot-diet.

Why do not all of the rats develop edema and why is the edema intermittent? It is difficult to explain why some of the rats on this low-protein-diet develop edema and others do not. There is no relation

Rat 321 Rat 319 Normal rat Rat 319

Fig. 4. Rats 321 and 319 are from the same litter. The photograph was made after the rats had been feeding on experimental diets for eight weeks. Rat 321 received the control diet with adequate protein in the form of pure casein. It has grown normally (as shown by the photograph and by the growth curve in figure 2) and later reproduced normally. Rat 319 received the low-protein-carrot-diet and at the time the photograph was made was showing the early signs of edema, as shown in both the side view and front view of the rat. Its cheeks puff out and its eyes are partly closed. The normal rat was selected from the stock rats for a comparison of the normal shape of the face with the edematous face. It is a much younger rat than rat 319 but was selected because it was about the same size as No. 319. A comparison of the size of the eyes and the width of the face is quite striking.

between age or weight and the development of edema, neither is one litter more liable to edema than another. All of the rats on this diet grow weak and are quite similar in showing all the other symptoms of malnutrition. A muscular wasting is evident in all and an atrophy of the testicles of the male is quite evident. No doubt histological work would reveal atrophy in all the tissues. But why should the tissues of one rat undergo changes which cause them to retain fluids to this excessive degree and those of another rat not undergo these same changes, when both rats are from the same litter, have the same weight, and have the same environment and are receiving equal amounts of the same kind of food? Neither is there any relation between the amounts of food consumed and the development of edema; the food consumption varies among the rats that develop edema just as it varies among the rats that do not develop edema.

Another fact that is difficult to explain is the intermittency of the edema in many of the rats. This too is very irregular. Sometimes rats will show edema 6 or 8 weeks before their death. It disappears and may or may not return, or it may return and disappear several times. Very often rats will show an extreme edema, such as has been described as a "blister" on the chest and very often this disappears almost entirely in 12 to 24 hours, without any change in diet. Why do the tissues retain the fluid at one time and then change so suddenly? Could it be, perhaps, that the absence from the diet of sufficient amount of certain specific substances, whose absence is responsible for the development of the edema, causes the tissues generally or certain specific organs to undergo changes (as they do in actual starvation) which leads to the liberation of these specific substances and in turn to the temporary disappearance of the edema? No doubt a clearer knowledge of the tissue changes, that are responsible for the retention of the fluids, would throw some light on the subject.

How does the acid content of the diet influence the edema? An attempt was made to determine more accurately the changes that take place in the tissues to enable them to retain water to the striking degree that occurred in these edematous rats. As acidosis is a suggested cause of edema this was one of the first factors to be considered. The ordinary methods for determining this condition seemed difficult to apply in such small animals for it would be difficult to obtain sufficient blood without sacrificing the animal, which course did not seem practical in this stage of the work. As an indicative measure a diet was made using salt mixture VI instead of salt mixture II. Salt mixture VI has

Fig. 5. Rat 302 is a typical case of the edema in the form of a "blister" on the chest. The hair of the rat was moistened with water and brushed down in order to give a better view of the large amount of fluid collected subcutaneously on the chest of the rat. This rat was very weak at the time the photograph was made and died about 12 hours later, but other rats with the same type of edema and of the same degree were cured by the administration of a diet of the same caloric but with an adequate protein content.

slightly more of the acid salt $Ca(H_2PO_4)_2$ in place of some of the calcium lactate as in salt mixture II. With the exception of this small change in the acid content this new diet was identical with diet XII (see table 3). As a control diet XII (a) was used. Of the rats fed this diet with increased acid content, 88 per cent (8 out of 9 rats) developed edema whereas on the control diet only 50 per cent (3 out of 6 rats) developed edema. It should be noted, however, that when diet XII (a) was fed earlier, as recorded in table 3, $66\frac{2}{3}$ per cent of the rats developed edema, a somewhat greater percentage than occurred in this experiment. These results would indicate that the increased acid content of the diet so alters or injures the tissues as to make them more likely to retain water. That the acid itself is not the cause of the edema is shown by the fact that one of the rats that had developed edema on this more acid diet was cured by putting him on a diet with the same acid content but with 18 per cent pure casein replacing 18 per cent cornstarch. This rat was not only cured but in the succeeding 5 weeks increased in body weight 26 grams. This strengthens the evidence for the curative value of adequate protein in cases of edema of this type since it is here successful in curing the edema with the adverse condition of the higher acid content present.

The relation of the kidney to the protein deficiency. There is some indication that the kidney cells are injured and therefore fail to eliminate the water. Although very little work was done on the kidney tissues, sections were made of a few kidneys and some albumin found in the tubules. Attempts were made to test the urine. It was difficult to collect uncontaminated urine from the rats so that the urine was collected, after death, from the urethra (where urine remained in the bladder) by slight pressure on the bladder. Positive tests for albumin were obtained but when the normal rats were killed and the urine collected in the same manner and tested, positive results were also found which made the above experiments of no value. And yet it seems probable that the albumin in the tubules would follow the current of urine into the bladder.

In this connection it is interesting to note the work of Epstein (32), who recommends a diet high in well-selected proteins and low in carbohydrates and fats, in cases of edema in certain types of chronic nephritis. He further suggests that chronic parenchymatous nephritis might be genetically a disorder of nutrition. Allbutt (33) reports a case of renal dropsy cured by a high protein diet.

It is reported by Captain McCay (34) that renal disease is more common among the population of Bengal than among the Europeans who consume more protein than the Bengali.

One of the long recognized results of a high protein diet is the impairment of kidney cells. Chittenden (35) emphasizes the importance of limiting the "daily intake of protein food to as low a level as is consistent with the true needs of the body, in those cases where the kidneys are at all enfeebled or where it seems desirable to exercise due precaution as a possible means of prevention." From the evidence here given it would seem that there is equally as great danger of renal impairment from a low protein diet. This follows very reasonably for certainly there is a weakening of all the tissues, quite evident in the general muscular weakness of the animal which is certainly due to a specific protein starvation of the cells.

It is reported (3) that often edema did not occur among people when on the restricted war diet, until they did hard work. It is further reported that rest in bed along with dietary changes was very beneficial. This would indicate that muscular work is a predisposing factor to the edema, which seems very probable. If the tissues of the body are not receiving enough food, certainly muscular work would relatively diminish the amount of food. Furthermore, during muscular work lactic acid is formed and according to Fischer (36) acid renders the tissues able to hold more water.

CONCLUSIONS

1. When young rats are fed diets composed largely of carrots and with carrots as the only source of protein, a large percentage of the rats develop edema.

2. Fats or fat-soluble-vitamine do not prevent the occurrence or decrease the percentage of edema in rats, even if 10 per cent of the calorie value of the diet is made up of butter-fat.

3. The water-soluble-vitamine does not prevent the occurrence or decrease the percentage of edema in rats fed the low-protein-diet.

4. Salts do not play any appreciable rôle in the production of this type of edema, for even when the salt content is doubled there is no noticeable effect upon the occurrence of edema.

5. The water intake of the animal which is feeding on the low-protein-carrot-diet decidedly influences the development of the edema. Edema develops more frequently, is more severe and develops sooner on a wet diet than on a dry diet.

6. When a sufficient amount of an adequate protein is added to the low-protein-carrot-diet, in place of an equivalent amount of cornstarch, edema is not only averted in rats feeding on this diet but the rats show normal growth and reproduction.

7. That the edema does not develop as a result of some toxic substance in the carrots is shown by the fact that rats fed on the control diet with the same carrot content as the low-protein-carrot-diet but with adequate protein did not develop edema and showed normal well-being.

8. When the acid content of the diet was increased the percentage of rats developing edema was increased from 50 per cent to 88 per cent. That the acid in this diet was not the chief etiological factor of the edema is shown by the fact that one rat on this diet was cured and made to grow normally on the diet with no change in the acid content but with an adequate protein supply replacing an equivalent amount of corn starch in the diet.

9. The edema manifested in these rats is not due to simple starvation, or low caloric intake, for when rats are fed a diet, including 1 gram of adequate protein each day, of the same caloric value as the diet fed the edematous rats, they do not develop edema.

10. This edema can be successfully cured by supplying the diet with adequate protein. Edema was produced and cured three times in one rat by alternating low-protein-diet (edema-producing) with an adequate protein diet.

11. These findings warrant the general conclusion that if it is necessary to limit the amount of protein in a diseased condition or in a period of national economic stress (as was necessary in some of the European countries during the recent war), it is advisable to administer the low protein diet in a form free from excess of water and any acid producing foods. Symptoms of developing edema must be looked for and adequate protein supplied immediately to effect a cure.

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Note 2. The wheat germ used in all of these experiments was furnished by the Washburn-Crosby Milling Co.

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